NON-PROVISIONAL
UTILITY PATENT APPLICATION
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First Named Inventor: Cesare Peschle
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Transmitted herewith for filing is the **non-provisional** utility patent application entitled:

COMPOSITIONS AND METHODS FOR USE IN AFFECTING HEMATOPOIETIC STEM CELL POPULATIONS IN MAMMALS

which is:

an **[X]** Original; or

a **[ ]** Continuation, **[ ]** Divisional, or **[ ]** Continuation-in-part (CIP)
of prior Application No. _____/_____, filed _____.

[X] This non-provisional patent application is based on Provisional Patent Application No. 60/087,153, filed May 29, 1998.

Enclosed are:

- **[X]** Specification (including Abstract) and claims: 70 pages.
- **[X]** Newly unexecuted Declaration (copy).
- **[ ]** Copy of Declaration from prior application.
- **[ ]** Separate Power of Attorney (including 37 CFR 3.73(b) statement, if applicable).
- **[X]** 10 sheets of drawings (formal) plus one copy.
- **[ ]** Microfiche computer program (Appendix).
- **[ ]** Nucleotide and/or Amino Acid Sequence Submission, including:
  [ ] Computer readable copy **[ ]** Paper Copy **[ ]** Verified Statement.
- **[ ]** Under PTO-1595 cover sheet, an assignment of the invention.
- **[ ]** Certified copy of _____ Application No. _____, filed _____, is filed:
  [ ] herewith or **[ ]** in prior application _____.
- **[X]** Verified Statement Claiming Small Entity Status under 37 CFR 1.9 and 1.27.
  - **[ ]** was filed in the prior non-provisional application, and such status is still proper and desired (37 CFR 1.28(a));
  - **[X]** is enclosed herewith; **[ ]** is no longer desired.
- **[ ]** Preliminary Amendment.
- **[ ]** Information Disclosure Statement, PTO-1449, and cited references.
- **[ ]** Other:

The filing fee has been calculated as shown below:

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CLAIMS	NO. FILED	NO. EXTRA	BASIC FEE: $380.			BASIC FEE: $760	
Total	70-20 =	50	X9	$450.	OR	X18	$
Independent	10-3=	7	X39	$273.	OR	X78	$
Multiple Dependent Claims Present ____			$130	$	OR	$260	$
			TOTAL	**$-0-**	OR	**TOTAL**	**$**

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[X] If the filing of any paper during the prosecution of this application requires an extension of time in order for the paper to be timely filed, applicant(s) hereby petition(s) for the appropriate extension of time pursuant to 37 C.F.R. §1.136(a).

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May 28, 1999
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Enclosures

Attorney's Docket No. 9855-26U1
(OTT-3038-1)

Applicant or Patentee: **Peschle *et al.***
Application or Patent No.: **Not Yet Assigned**
Filed or Issued: **Herewith**
For: **COMPOSITIONS AND METHODS FOR USE IN AFFECTING HEMATOPOIETIC STEM CELL POPULATIONS IN MAMMALS**

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

NAME OF ORGANIZATION: Thomas Jefferson University
ADDRESS OF ORGANIZATION: 1020 Walnut Street
Philadelphia, PA 19107

TYPE OF ORGANIZATION:

[X] University or other institution of higher education.
[ ] Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)).
[ ] Nonprofit scientific or educational under statute of state of the United States of America.
Name of State _____
Citation of Statute _____
[ ] Would qualify as tax exempt under Internal Revenue Code (26 USC 50 (a) and 501(c)(3) if located in the United States of America.
[ ] Would qualify as nonprofit scientific or educational under statute of state of United States of America if located in the United States of America.
Name of State _____
Citation of Statute _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, with regard to the invention of the above-identified patent or patent application.

I hereby declare that U.S. rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under

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*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27).

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[ ]Individual [ ]Small Business Concern [ ]Nonprofit Organization

FULL NAME _____
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[ ]Individual [ ]Small Business Concern [ ]Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in the status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

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SIGNATURE _____ DATE: 5/28/99

TITLE OF THE INVENTION

Compositions and Methods for Use in Affecting
Hematopoietic Stem Cell Populations in Mammals

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority, under 35 U.S.C. § 119(e), to U.S. Provisional Application No. 60/087,153, filed on May 29, 1998, which is hereby incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Hematopoiesis in mammals is maintained by a pool of self-renewing hematopoietic stem cells (HSCs) (Ogawa, 1993, Blood 81:2844-2853). HSCs feed into lineage(s)-committed undifferentiated hematopoietic progenitor cells (HPCs) with little or no self-renewal capacity (Ogawa, 1993, Blood 81:2844-2853). The HPCs in turn generate morphologically recognizable differentiated precursors and terminal cells circulating in peripheral blood.

Human HSCs are identified on the basis of their capacity for long-term hematopoietic repopulation *in vitro* and *in vivo*. Specifically, *in vitro* repopulation of an irradiated allogeneic stromal adherent layer in long term culture (LTC) of Dexter type has been observed. In Dexter type LTC, primitive HPCs and HSCs are assessed as five to eight week and twelve week LTC initiating cells (LTC-ICs; Sutherland et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:3584-3588; Valtieri et al., 1994, Cancer Res. 54:4398-4404; Hao et al., 1996, Blood 88:3306-3313), or cobblestone area forming cells (CAFCs; Breems et al., 1996, Blood 87:5370-5378). Particularly, short term repopulating primitive HPCs have been identified in five to eight week LTC (Sutherland et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:3584-3588; Larochelle et al., 1996, Nature Med. 2:1329-1337), whereas long-term repopulating putative HSCs have been identified in twelve week LTC (Hao et al., 1996, Blood 88:3306-3313). Moreover, *in vivo* repopulation of severe combined immunodeficiency (SCID) mice at

two months (Nolta et al., 1994, Blood 83:3041-3047) or non-obese diabetic SCID (SCID-NOD) mice at one and a half months (Bock et al., 1995, J. Exp. Med. 182:2037-2043) after irradiation and HSC injection has been observed.

In murine embryonic life (day 7.5 of gestation), a close developmental association of the hematopoietic and endothelial lineages takes place in the yolk sack blood islands, leading to the hypothesis that the two lineages share a common ancestor referred to as the hemoangioblast (Flamme et al., 1992, Development 116:435-439; Risau et al., 1995, Ann. Rev. Cell. Dev. Biol. 11:73-91).

Vascular endothelial growth factor (VEGF) and one of its receptors, VEGFRII termed Flk1 in mice and KDR in humans, play a key role in early hemoangiogenesis. In fact, Flk1$^-$ knock-out mice are unable to form blood islands and blood vessels (Shalaby et al., 1995, Nature 376:62-66). Differentiated murine embryonic stem cells treated with VEGF and the ligand for c-kit receptor at the embryoid stage give rise to primitive blast cells which generate the various hematopoietic lineages (Kennedy et al., 1997, Nature 386:488-492; Kabrun et al., 1997, Development 124:2039-2048): these data suggest a role for VEGF at the level of primitive HPCs in murine embryonic hematopoiesis. There are no data concerning the effect of expression or the function of KDR in human embryonic/fetal HSCs.

In post-fetal life, the VEGF/KDR system plays an important role in the endothelial lineage. Indeed, KDR and CD34 antigens are expressed on progenitors of human adult endothelial cells (Ashara et al., 1997, Science 275:964-967). Again, there are no data concerning the effect of expression or the function of KDR in human post-fetal HSCs, particularly long-term repopulating HSCs. Most studies have focused on examination of the effect of VEGF on partially purified HPCs. The results of these studies suggest that VEGF exerts an enhancing or inhibitory effect on bone marrow (BM) HPC colony formation stimulated by diverse hematopoietic growth factors (HGFs; Broxmeyer et al., 1995, Int. J. Hematol. 62:203-215) and a stimulatory effect on hematopoietic cells in normal mice (Gabrilovich et al., 1998, Blood 92:4150-4166). In addition, KDR mRNA is expressed in cord blood (CB) and BM partially purified

HPCs, while VEGF does not affect CB HPC colony formation but exerts an anti-apoptotic action on irradiated HPCs (Katoh et al., 1995, Cancer Res. 55:5687-5692).

There is a need in the art for efficient methods of purifying and characterizing long term repopulating HSCs and for methods of *ex vivo* expansion of these cells. In addition, there is a need in the art for methods of treating a variety of diseases using HSCs. The present invention satisfies these needs.

BRIEF SUMMARY OF THE INVENTION

The invention includes a method of obtaining a cell population enriched for long-term repopulating human hematopoietic stem cells. The method comprises obtaining a population of cells from human hematopoietic tissue and isolating a population of KDR^+ cells therefrom, thereby obtaining a cell population enriched for long-term repopulating human hematopoietic stem cells.

In one aspect, the human hematopoietic tissue is selected from the group consisting of pre-embryonic hematopoietic tissue, embryonic hematopoietic tissue, fetal hematopoietic tissue, and post-natal hematopoietic tissue.

In another aspect, the embryonic hematopoietic tissue is selected from the group consisting of yolk sac, and embryonic liver.

In yet another aspect, the fetal hematopoietic tissue is selected from the group consisting of fetal liver, fetal bone marrow and fetal peripheral blood.

In a further aspect, the post-natal hematopoietic tissue is selected from the group consisting of cord blood, bone marrow, normal peripheral blood, mobilized peripheral blood, hepatic hematopoietic tissue, and splenic hematopoietic tissue.

In yet a further aspect, the KDR^+ cells are isolated using a reagent which specifically binds KDR.

In one aspect, the reagent is an antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

In another aspect, the antibody is a monoclonal antibody.

In yet another aspect, the monoclonal antibody is 260.4.

In a further aspect, the KDR^+ cells are isolated using a conjugated vascular epithelial growth factor or a molecule derived therefrom.

In yet a further aspect, the cells are starvation resistant long-term repopulating human hematopoietic stem cells.

The invention includes an enriched population of long-term repopulating human hematopoietic stem cells obtained using a method of obtaining a cell population enriched for long-term repopulating human hematopoietic stem cells. The method comprises obtaining a population of cells from human hematopoietic tissue and isolating a population of KDR^+ cells therefrom, thereby obtaining a cell population enriched for long-term repopulating human hematopoietic stem cells. The invention also includes a cell isolated using this method. The invention also includes the cell isolated using this method wherein the cell comprises an isolated nucleic acid.

In one aspect, the cell comprising an isolated nucleic acid comprises an isolated nucleic acid selected from the group consisting of a nucleic acid encoding adenosine deamininase, a nucleic acid encoding β-globin, a nucleic acid encoding multiple drug resistance, an antisense nucleic acid complementary to a human immunodeficiency virus nucleic acid, an antisense nucleic acid complementary to a nucleic acid encoding a cell cycle gene, and an antisense nucleic acid complementary to a nucleic acid encoding an oncogene.

In another aspect, the isolated nucleic acid is operably linked to a promoter/regulatory sequence.

In even another aspect, the promoter/regulatory sequence is selected from the group consisting of a retroviral long terminal repeat, and the cytomegalovirus immediate early promoter.

The invention includes a method of obtaining a purified population of long-term repopulating human hematopoietic stem cells. The method comprises obtaining a population of cells from human hematopoietic tissue, isolating a population of hematopoietic progenitor cells therefrom, and isolating a population of KDR^+ cells

from the population of hematopoietic progenitor cells, thereby obtaining a purified population of long-term repopulating human hematopoietic stem cells.

In one aspect, the human hematopoietic tissue is selected from the group consisting of pre-embryonic hematopoietic tissue, embryonic hematopoietic tissue, fetal hematopoietic tissue, and post-natal hematopoietic tissue.

In another aspect, the embryonic hematopoietic tissue is selected from the group consisting of yolk sac, and embryonic liver.

In yet another aspect, the fetal hematopoietic tissue is selected from the group consisting of fetal liver, fetal bone marrow and fetal peripheral blood.

In a further aspect, the post-natal hematopoietic tissue is selected from the group consisting of cord blood, bone marrow, normal peripheral blood, mobilized peripheral blood, hepatic hematopoietic tissue, and splenic hematopoietic tissue.

In yet a further aspect, the hematopoietic progenitor cells are isolated using at least one method selected from the group consisting of isolation of cells expressing an early marker using antibodies specific for said marker, isolation of cells not expressing a late marker using antibodies specific for said late marker, isolation of cells based on a physical property of said cells, and isolation of cells based on a biochemical/biological property of said cells.

In another aspect, the early marker is selected from the group consisting of CD34, Thy-1, c-kit receptor, flt3 receptor, AC133, vascular endothelial growth factor receptor I, vascular endothelial growth factor receptor III, Tie1, Tek, and basic fibroblast growth factor receptor.

In yet another aspect, the late marker is a lineage (lin) marker.

In a further aspect, the early marker is CD34.

In even a further aspect, the hematopoietic progenitor cells are obtained from the hematopoietic tissue using an antibody which specifically binds CD34 to select a population of $CD34^+$ hematopoietic progenitor cells.

In another aspect, the population of KDR^+ cells is isolated from the population of $CD34^+$ hematopoietic progenitor cells using an antibody which specifically binds KDR.

In yet another aspect, the antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

In even yet another aspect, the antibody is a monoclonal antibody.

In a further aspect, the monoclonal antibody is 260.4.

In even a further aspect, the cells are starvation resistant human hematopoietic stem cells.

The invention includes an isolated purified population of long-term repopulating human hematopoietic stem cells obtained by a method of obtaining a purified population of long-term repopulating human hematopoietic stem cells. The method comprises obtaining a population of cells from human hematopoietic tissue, isolating a population of hematopoietic progenitor cells therefrom, and isolating a population of KDR^+ cells from the population of hematopoietic progenitor cells, thereby obtaining a purified population of long-term repopulating human hematopoietic stem cells. The invention also includes a cell obtained by this method. The invention further includes a cell obtained by this method wherein the cell comprises an isolated nucleic acid.

The one aspect, the isolated nucleic acid is selected from the group consisting of a nucleic acid encoding adenosine deaminase, a nucleic acid encoding β-globin, a nucleic acid encoding multiple drug resistance, an antisense nucleic acid complementary to a human immunodeficiency virus nucleic acid, an antisense nucleic acid complementary to a nucleic acid encoding a cell cycle gene, and an antisense nucleic acid complementary to a nucleic acid encoding an oncogene.

In another aspect, the isolated nucleic acid is operably linked to a promoter/regulatory sequence.

In yet another aspect, the promoter/regulatory sequence is selected from the group consisting of a retroviral long terminal repeat, and the cytomegalovirus immediate early promoter.

In a further aspect, the hematopoietic progenitor cells are obtained from said hematopoietic tissue using antibody which specifically binds CD34 to select a population of $CD34^-$ cells.

In an even further aspect, the hematopoietic progenitor cells are obtained from said population of $CD34^-$ cells using antibody which specifically binds lin to select a population of $CD34^-lin^-$ cells.

In another aspect, the population of KDR^+ cells is isolated from the population of $CD34^-lin^-$ cells using an antibody which specifically binds KDR.

In yet another aspect, the antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

In even another aspect, the antibody is a monoclonal antibody.

In a further aspect, the monoclonal antibody is 260.4.

The invention includes an isolated purified population of long-term repopulating human hematopoietic stem cells obtained by a method of obtaining a purified population of long-term repopulating human hematopoietic stem cells. The method comprises obtaining a population of cells from human hematopoietic tissue, isolating a population of hematopoietic progenitor cells therefrom, and isolating a population of KDR^+ cells from the population of hematopoietic progenitor cells, thereby obtaining a purified population of long-term repopulating human hematopoietic stem cells. The invention also includes a cell obtained by this method.

The invention further includes the cell obtained by this method wherein the cell comprises an isolated nucleic acid.

In one aspect, the isolated nucleic acid is selected from the group consisting of a nucleic acid encoding adenosine deaminase, a nucleic acid encoding β-globin, a nucleic acid encoding multiple drug resistance, an antisense nucleic acid complementary to a human immunodeficiency virus nucleic acid, an antisense nucleic

acid complementary to a nucleic acid encoding a cell cycle gene, and an antisense nucleic acid complementary to a nucleic acid encoding an oncogene.

In another aspect, the isolated nucleic acid is operably linked to a promoter/regulatory sequence.

In yet another aspect, the promoter/regulatory sequence is selected from the group consisting of a retroviral long terminal repeat, and the cytomegalovirus immediate early promoter.

The invention includes a method of expanding a population of long-term repopulating human hematopoietic stem cells. The method comprises obtaining a population of cells from human hematopoietic tissue, isolating a population of KDR^+ hematopoietic stem cells therefrom, and incubating the population of KDR^+ cells with vascular endothelial growth factor, thereby expanding the population of long-term repopulating human hematopoietic stem cells.

In one aspect, the method further comprises incubating the population of KDR^+ cells with at least one growth factor.

In another aspect, the growth factor is selected from the group consisting of flt3 receptor ligand, kit receptor ligand, thrombopoietin, basic fibroblast growth factor, interleukin 6, interleukin 11, interleukin 3, granulomonocytic colony-stimulatory factor, granulocytic colony-stimulatory factor, monocytic colony-stimulatory factor, erythropoietin, angiopoietin, and hepatocyte growth factor.

The invention also includes an isolated purified population of long-term repopulating human hematopoietic stem cells obtained by this method.

The invention further includes a cell obtained using this method.

In one aspect, the cell comprises an isolated nucleic acid.

In another aspect, the isolated nucleic acid is selected from the group consisting of a nucleic acid encoding adenosine deaminase, a nucleic acid encoding β-globin, a nucleic acid encoding multiple drug resistance, an antisense nucleic acid complementary to a human immunodeficiency virus nucleic acid, an antisense nucleic

acid complementary to a nucleic acid encoding a cell cycle gene, and an antisense nucleic acid complementary to a nucleic acid encoding an oncogene.

In yet another aspect, the isolated nucleic acid is operably linked to a promoter/regulatory sequence.

In a further aspect, the promoter/regulatory sequence is selected from the group consisting of a retroviral long terminal repeat, and the cytomegalovirus immediate early promoter.

The invention includes a blood substitute comprising the progeny cells of an isolated purified population of long term repopulating human hematopoietic stem cells.

In one aspect, the progeny cells are selected from the group consisting of red blood cells, neutrophilic granulocytes, eosinophilic granulocytes, basophilic granulocytes, monocytes, dendritic cells, platelets, B lymphocytes, T lymphocytes, natural killer cells, and differentiated precursors thereof, and undifferentiated progenitors thereof.

The invention also includes a chimeric non-human mammal comprising at least one of an isolated and purified long-term repopulating human hematopoietic stem cell.

In one aspect, the cell is introduced into the mammal using a method selected from the group consisting of transplantation, and blastocyst injection.

In another aspect, the mammal is selected from the group consisting of a mouse, a rat, a dog, a donkey, a sheep, a pig, a horse, a cow, a non-human primate.

The invention includes a method of inhibiting rejection of a transplanted organ. The method comprises ablating the bone marrow of a transplant recipient and administering to the recipient a multi-lineage engrafting dose of an isolated and purified long-term repopulating human hematopoietic stem cell obtained from the hematopoietic tissue of the donor of said organ, thereby inhibiting rejection of a transplanted organ.

The invention includes a method of transplanting an autologous human hematopoietic stem cell in a human. The method comprises obtaining a population of cells from the hematopoietic tissue of a human and isolating a population of non-malignant hematopoietic stem cells therefrom, ablating the bone marrow of the human, and administering at least one isolated non-malignant hematopoietic stem cell to the human, thereby transplanting an autologous human hematopoietic stem cell in a human.

The invention also includes a method of isolating a KDR^+ cell. The method comprises selecting a cell expressing an antigen coexpressed with KDR, thereby isolating a KDR+ cell.

In one aspect, the coexpressed antigen is selected from the group consisting of a vascular endothelial growth factor receptor I, and a vascular endothelial growth factor receptor III.

The invention includes a method of isolating a KDR+ stem cell giving rise to at least one of a muscle cell, a hepatic oval cell, a bone cell, a cartilage cell, a fat cell, a tendon cell, and a marrow stroma cell. The method comprises isolating a KDR+ stem cell from hematopoietic tissue, thereby isolating a KDR+ stem cell giving rise to at least one of a muscle cell, a hepatic oval cell, a bone cell, a cartilage cell, a fat cell, a tendon cell, and a marrow stroma cell.

The invention includes a method of monitoring the presence of KDR+ stem cells in a human hematopoietic tissue in a human receiving therapy. The method comprises obtaining a sample of hematopoietic tissue from the human before, during and after the therapy, and measuring the number of KDR+ stem cells in the sample, thereby monitoring the presence of KDR+ stem cells in a human hematopoietic tissue obtained from a human receiving therapy.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1A is a graph depicting representative results on KDR expression and distribution of $CD34^+$ cells by flow cytometry. KDR expression (top

panels) detected by flow cytometry on bone marrow (BM), normal or mobilized peripheral blood (PB, MPB),and cord blood (CB) in $CD34^+$ cells. Cells gated on physical parameters were analyzed for specific and nonspecific (isotype-matched) antibody reactivity (greater than about 40,000 cells were analyzed). The percentage of $CD34^+KDR^+$ cells is indicated by numbers on the figure. Bottom left: This graph depicts representative gates for analysis and sorting of KDR^+ (KDR^{bright}), $KDR^{+/\pm}$ (KDR^{dim}) and KDR^- $CD34^+$ cells. A cord blood (CB) experiment is shown. The bottom right panel is an image of a gel depicting the RT-PCR analysis detecting the presence of KDR mRNA in $CD34^+KDR^+$ versus $CD34^+KDR^-$ CB sorted cell populations.

Figure 1B is a graph depicting representative results on the expression of KDR and relevant early hematopoietic antigens in electronically gated $CD34^+$ cells. Electronically gated $CD34^+$ cells (top panels) from BM, PB, MPB, and CB were analyzed for expression of KDR and several early hematopoietic antigens, as indicated.

Figure 2A is a graph depicting the *in vitro* HPC/HSC assays of $CD34^+KDR^+$ cells. Top panel depicts the HPCs in PB $CD34^+KDR^{+/\pm}$ and $CD34^+KDR^-$ cells assayed in cultures supplemented with a restricted (left) or large (right) spectrum of hematopoietic growth factors (HGFs). The bottom panels depict primary and secondary HPP-CFC colonies in PB $CD34^+KDR^+$ and $CD34^+KDR^-$ cells. Mean ± SEM from 4 independent experiments is disclosed.

Figure 2B is a graph depicting the PB $CD34^+$, $CD34^+KDR^-$, and $CD34^+KDR^{+/\pm}$ cell LTC (left panel): at 5, 8, and 12 weeks, supernatant and adherent cells were assayed for HPCs. The middle and right panels depict BM (middle) and CB (right) $CD34^+KDR^-$, $CD34^+KDR^+$ cell LTC analyzed for CAFC-derived colonies at 6, 9, and 12 weeks. Mean ± SEM from three experiments is disclosed.

Figure 2C is a graph depicting LDA of 12 week LTC-ICs/CAFCs in $CD34^+KDR^+$ cells. The top left panel depicts LTC-IC frequency in PB $CD34^+$, $CD34^+$ $KDR^{+/\pm}$, $CD34^+KDR^+$, and $CD34^+KDR^-$ cells. The mean ± SEM for five separate $VEGF^+$ or three separate $VEGF^-$ experiments is shown. The top right panel

depicts representative LDAs for PB $CD34^+$ and $CD34^+KDR^+$ cells (100 replicates for the lowest cell concentration (*e.g.*, 1 KDR^+ cell) and decreasing replicate numbers for increasing cell concentrations, *i.e.*, 50, 20, 10 wells with 2, 5, 10 KDR^+ cells, respectively. The bottom panels depict CAFC frequency in KDR^+ and KDR^- cells from BM (left) or CB (right). The mean $\pm$ SEM for three separate experiments is shown. The ** indicates that $p<0.01$ when compared to the $VEGF^-$ group. The symbol °° indicates that $p<0.01$ when compared to the other groups.

Figure 2D is a graph depicting the starvation of PB $CD34^+KDR^{+/\pm}$, or $CD34^+$ KDR^- cells in single cell FCS^- free liquid phase culture supplemented or not with VEGF (top): the percentage of cells that survived at day 21 (mean ± SEM from 3 separate experiments) is shown. The bottom panels depict the minibulk (2×10^3 cells/ml) PB $CD34^+KDR^{+/\pm}$ starvation culture supplemented with VEGF: the limiting dilution assay (LDA) of LTC-IC frequency in the approximately 25% cells surviving on day 5 and 25 is shown.

Figure 3A is a graph depicting representative results on the engraftment of BM $CD34^+KDR^+$ cells in NOD-SCID mice demonstrating the repopulating activity of 100 to 1,600 $CD34^+KDR^+$ cells in recipient mice. The positive and negative controls received $CD34^+$ and $CD34^+KDR^-$ cells, respectively (top left). The top panels depict human $CD34^+/CD45^+$ cell engraftment (left top panel) and $CD45^+$ cell dose-response (mean ± SEM, three mice/group, r=0.99) (right top panel). Dose-dependent engraftment was also observed in recipient PB and spleen. The bottom panels depict the expression of human hematolymphopoietic markers in a representative mouse injected with 1,600 $CD34^+KDR^+$ cells.

Figure 3B is a graph depicting representative results on the engraftment of BM $CD34^+KDR^+$ cells in NOD-SCID mice demonstrating the LDA of repopulating HSC frequency in $CD34^+KDR^+$ cells. Graded numbers of BM $CD34^+KDR^+$ cells were injected into recipient mice. The positive and negative controls received $CD34^+$ and $CD34^+KDR^-$ cells, respectively. The top panels depict human $CD45^+$ cells in BM of mice injected with 250, 50, 10 or 5 cells (3, 9, 6 and 6 mice per group, respectively)

(mean ± SEM). The bottom left panel depicts human HPCs in BM of the 4 engrafted mice injected with 5 cells (mean ± SEM) (left) and the LDA according to single hit Poisson statistics (right). The bottom right panel depicts the PCR analysis of human alpha-satellite DNA (867 bp band) in all scored colonies from a representative mouse that received 5 cells. The contents of the lanes are indicated in the figure, in addition lane 13 depicts a human DNA positive control, lane 14 depicts a no DNA template negative control, lane 15 comprises DNA from BM mononuclear cells of a nontransplanted mouse and M.W. indicates a lane comprising molecular weight markers.

Figure 3C is a graph depicting the expression of informative human hematolymphopoietic markers in a representative mouse receiving 6,000 CB $CD34^+KDR^+$ cells as described elsewhere herein.

Figure 4 is a graph (comprising three panels) depicting representative results on the engraftment of BM $CD34^+KDR^+$ cells in primary and secondary fetal sheep. The total estimated number of human $CD34^+$, $CD45^+$, glycophorin A^+ (GPA^+), and $CD7^+$ cells generated in primary fetal sheep recipients transplanted with $CD34^+KDR^{+/\pm}$ (middle panel) or $CD34^+KDR^-$ cells top panel) (mean ± SEM). The percentage of human $CD45^+$ cells and total HPCs in BM of secondary sheep fetuses is depicted in the bottom panel (mean ± SEM).

DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the discovery that VEGFRII (KDR) is a key functional marker for long-term repopulating human HSCs. The identification of HSCs expressing KDR (*i.e.*, KDR^+ HSCs) serves to facilitate the development of improved methodology for the purification and characterization of long-term repopulating HSCs. The identification of KDR^+ HSCs also serves to facilitate *ex vivo* expansion of purified HSCs by incubation of cells from hematopoietic tissue with VEGF combined with other hematopoietic growth factors (HGFs). Generation of chimeric animals (at the

somatic level) through human HSC injection into the animal blastocyst generates human hematopoietic cells in this animal *in vivo*.

At a clinical level, purified KDR^+ HSCs serve as key innovative tools for allogeneic or autologous HSC transplantation, as applied in leukemia/lymphoma, solid tumors, hematopoietic diseases and autoimmune disorders, and for HSC-based gene therapy for treatment of a large spectrum of hereditary of acquired disorders affecting hematopoiesis and/or lymphopoiesis (*e.g.,* AIDS). In addition, following *in vitro* expansion and differentiation of purified KDR^+ HSCs, the KDR^+ HSC progeny, for example, red blood cells, granulocytes and/or platelets, are useful in transfusion medicine.

The invention thus includes a method of obtaining a cell population enriched for long-term repopulating human hematopoietic stem cells. The method comprises obtaining a population of cells from human hematopoietic tissue. From the cells obtained from the hematopoietic tissue, cells expressing KDR on the surface of the cells are then isolated. In one embodiment, the KDR expressing cells are isolated using monoclonal antibody 260.4. However, the present invention should not be construed to be limited to isolation of KDR^+ cells using any particular antibody. Rather, the present invention encompasses using any antibody which specifically binds KDR to isolate KDR^+ cells including polyclonal antibody.

The invention includes a population of cells obtained using this method.

The invention also includes a method of obtaining a cell population enriched for long-term repopulating human hematopoietic stem cells wherein KDR^+ cells are isolated using a conjugated vascular epithelium growth factor. This method simply capitalizes on the affinity of the KDR-VEGF receptor-ligand interaction to select cells expressing KDR on their surfaces by binding such cells, via the KDR present on the surface of the cell, to VEGF conjugated to, for example, a solid support matrix. Thus, the VEGF-conjugate can be used to affinity-purify the KDR expressing cells by standard methods well-known in the art.

The invention includes a population of cells obtained using this method.

One skilled in the art would appreciate, based upon the disclosure provided herein, that the KDR^+ cell fraction will not be comprised solely of long-term repopulating HSCs; instead, the fraction may include other cells such as megakaryocytes, endothelial cells, and the like, which express KDR but which are not HSCs. Preferably, these cells may be removed from the KDR^+ HSCs by various methods well-known in the art based on the physical, biochemical, immunological, and/or morphological differences between these cells and the KDR^+ undifferentiated hematopoietic progenitors and stem cells of interest. However, for purposes of the present invention, the non-HSC but KDR+ cells need not be removed from the KDR+ fraction isolated from human hematopoietic tissue.

Human hematopoietic tissue includes, but is not limited to, pre-embryonic, embryonic, fetal, and post-natal hematopoietic tissue. The embryonic hematopoietic tissue includes, for example, yolk sac and embryonic liver. Fetal hematopoietic tissue includes, but is not limited to, fetal liver, fetal bone marrow, and fetal peripheral blood. The post-natal hematopoietic tissue, in turn, includes cord blood, bone marrow, hepatic hematopoietic tissue, splenic hematopoietic tissue, and peripheral blood, both normal and mobilized.

The invention also includes a method of obtaining an enriched population of long-term repopulating HSCs that is starvation resistant. Starvation resistant cells are obtained by growing the KDR+ cells in mini-bulk culture under starvation conditions as described elsewhere herein. Starvation resistant cells obtained following culture constitute much fewer cell than are originally placed in serum-free culture in the absence of any HGF treatment, except for VEGF addition. However, the resulting starvation-resistant cells comprise a much higher percentage of putative HSCs than an otherwise identical population of cells that are not grown under identical conditions, therefore, putative HSCs are further enriched in the KDR+ fraction as a result of the starvation selection. The particular conditions for starvation culture are set forth elsewhere herein. One skilled in the art, based upon the disclosure provided herein, would appreciate that the particular conditions, *e.g.*, the precise number of days,

may be varied so long as serum and HGFs are not added into the medium in any significant amount. The resultant starvation resistant cells, which are enriched for *in vitro* long-term repopulating HSCs, may then be used in a wide variety of applications as described elsewhere herein.

The invention includes a cell obtained by the above-disclosed method of obtaining a cell population enriched for long-term repopulating human hematopoietic stem cells.

Further, the invention includes a cell obtained using this method wherein the cell comprises an isolated nucleic acid. The nucleic acid may be introduced into the cell using any method for introducing a nucleic acid into a cell and such methods are well-known in the art and are described, for example, in Sambrook et al. (1989, In: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York), and Ausubel et al. (1997, In: Current Protocols in Molecular Biology, Green & Wiley, New York). These methods include, but are not limited to, calcium phosphate precipitation transfection, DEAE dextran transfection, electroporation, microinjection, liposome-mediated transfer, chemical-mediated transfer, ligand-mediated transfer, and recombinant viral vector transfer, and the like.

The nucleic acid which may be transfected and/or transducted into the cell includes a nucleic acid such as that encoding adenosine deaminase, β-globin, and multidrug resistance. Thus, the cell may, if the nucleic acid is expressed, be used to provide the protein encoded thereby to the cell and/or to the extracellular milieu. The present invention should not be construed to be limited to these particular nucleic acids. Instead, a wide variety of nucleic acids encoding a plethora of proteins may be transfected into the cell of the invention. Thus, the invention should be construed to include nucleic acid products which are useful for the treatment of various disease states in a mammal. Such nucleic acids and associated disease states include, but are not limited to: DNA encoding glucose-6-phosphatase, associated with glycogen storage deficiency type 1A; DNA encoding phosphoenolpyruvate-carboxykinase, associated with Pepck deficiency; DNA encoding galactose-1 phosphate uridyl transferase,

associated with galactosemia; DNA encoding phenylalanine hydroxylase, associated with phenylketonuria; DNA encoding branched chain α-ketoacid dehydrogenase, associated with Maple syrup urine disease; DNA encoding fumarylacetoacetate hydrolase, associated with tyrosinemia type 1; DNA encoding methylmalonyl-CoA mutase, associated with methylmalonic acidemia; DNA encoding medium chain acyl CoA dehydrogenase, associated with medium chain acetyl CoA deficiency; DNA encoding ornithine transcarbamylase, associated with ornithine transcarbamylase deficiency ; DNA encoding argininosuccinic acid synthetase, associated with citrullinemia; DNA encoding low density lipoprotein receptor protein, associated with familial hypercholesterolemia; DNA encoding UDP-glucouronosyltransferase, associated with Crigler-Najjar disease; DNA encoding adenosine deaminase, associated with severe combined immunodeficiency disease; DNA encoding hypoxanthine guanine phosphoribosyl transferase, associated with Gout and Lesch-Nyan syndrome; DNA encoding biotinidase, associated with biotinidase deficiency; DNA encoding β-glucocerebrosidase, associated with Gaucher disease; DNA encoding β-glucuronidase, associated with Sly syndrome; DNA encoding peroxisome membrane protein 70 kDa, associated with Zellweger syndrome; DNA encoding porphobilinogen deaminase, associated with acute intermittent porphyria; DNA encoding $\alpha_1$antitrypsin for treatment of α-1 antitrypsin deficiency (emphysema); DNA encoding erythropoietin for treatment of anemia due to thalassemia or to renal failure; and, DNA encoding insulin for treatment of diabetes. Such DNAs and their associated diseases are reviewed in Kay et al. (1994, T.I.G. 10:253-257) and in Parker and Ponder (1996 , "Gene Therapy for Blood Protein Deficiencies," In: Gene Transfer in Cardiovascular Biology: Experimental Approaches and Therapeutic Implications, Keith and March, eds.).

One skilled in the art would appreciate, based upon the disclosure provided herein, that a human long-term repopulating hematopoietic stem cell able to engraft a recipient which cell comprises a nucleic acid is useful for gene therapy. That is, such a stem cell would, when introduced into an animal, express the nucleic acid thereby providing a method of producing a protein thus correcting a genetic defect in a

cell, encode a protein which is not otherwise present in sufficient and/or functional quantity such that it corrects a genetic defect in the cell, and/or encodes a protein which is useful as a therapeutic in the treatment or prevention of a particular disease condition or disorder or symptoms associated therewith. Thus, long-term repopulating human hematopoietic stem cells are useful therapeutics allowing the expression of an isolated nucleic acid present in such cell.

The invention also includes a cell transfected with an antisense nucleic acid complementary to a nucleic acid encoding a retrovirus such as human immunodeficiency virus, a cell cycle gene, and an oncogene. One skilled in the art would appreciate, based upon the disclosure provided herein, that under certain circumstances, it is useful to inhibit expression of a nucleic acid. In this regard, certain molecules, including antisense nucleic acids and ribozymes, are useful in inhibiting expression of a nucleic acid complementary thereto.

Antisense molecules and their use for inhibiting gene expression are well known in the art (*see, e.g.,* Cohen, 1989, In: Oligodeoxyribonucleotides, Antisense Inhibitors of Gene Expression, CRC Press). Antisense nucleic acids are DNA or RNA molecules that are complementary, as that term is defined elsewhere herein, to at least a portion of a specific mRNA molecule (Weintraub, 1990, Scientific American 262:40). In the cell, antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule thereby inhibiting the translation of genes.

The use of antisense methods to inhibit the translation of genes is known in the art, and is described, for example, in Marcus-Sakura, 1988, Anal. Biochem. 172:289. Such antisense molecules may be provided to the cell via genetic expression using DNA encoding the antisense molecule as taught by Inoue, 1993, U.S. Patent No. 5,190,931 (incorporated by reference herein in its entirety).

Alternatively, antisense molecules of the invention may be made synthetically and then provided to the cell. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and introduced into a target cell. Synthetic antisense molecules contemplated by the invention include

oligonucleotide derivatives known in the art which have improved biological activity compared to unmodified oligonucleotides (*see* Cohen, *supra*; Tullis, 1991, U.S. Patent No. 5,023,243, incorporated by reference herein in its entirety).

Ribozymes are another nucleic acid that may be transfected into the cell to inhibit nucleic acid expression in the cell. Ribozymes and their use for inhibiting gene expression are also well known in the art (*see, e.g.,* Cech et al., 1992, J. Biol. Chem. 267:17479-17482; Hampel et al., 1989, Biochemistry 28:4929-4933; Eckstein et al., International Publication No. WO 92/07065; Altman et al., U.S. Patent No. 5,168,053, incorporated by reference herein in its entirety). Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences encoding these RNAs, molecules can be engineered to recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988, J. Amer. Med. Assn. 260:3030). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes, namely, tetrahymena-type (Hasselhoff, 1988, Nature 334:585) and hammerhead-type. Tetrahymena-type ribozymes recognize sequences which are four bases in length, while hammerhead-type ribozymes recognize base sequences 11-18 bases in length. The longer the sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating specific mRNA species, and 18-base recognition sequences are preferable to shorter recognition sequences which may occur randomly within various unrelated mRNA molecules.

Ribozymes useful for inhibiting the expression of the proteins of interest may be designed by incorporating target sequences into the basic ribozyme structure which are complementary to the mRNA sequence of the nucleic acid encoding the protein of interest. Ribozymes targeting an immunodeficiency virus nucleic acid, a cell cycle gene, and an oncogene may be synthesized using commercially available reagents

(Applied Biosystems, Inc., Foster City, CA) or they may be expressed from DNA encoding them.

The invention includes a cell comprising an isolated nucleic acid wherein the nucleic acid is operably linked to a promoter/regulatory sequence. Accordingly, expression of the nucleic acid in cells which do not normally express the nucleic acid may be accomplished by transfecting the cell with a nucleic acid operably linked to a promoter/regulatory sequence which serves to drive expression of the nucleic acid. Many promoter/regulatory sequences useful for driving constitutive expression of a gene are available in the art and include, but are not limited to, for example, the cytomegalovirus immediate early promoter enhancer sequence, the SV40 early promoter, the Rous sarcoma virus promoter, and the like. Inducible and tissue specific expression of the nucleic acid operably linked thereto may be accomplished by placing the nucleic acid under the control of an inducible or tissue specific promoter/regulatory sequence. Examples of tissue specific or inducible promoter/regulatory sequences which are useful for this purpose include, but are not limited to the MMTV long terminal repeat (LTR) inducible promoter, and the SV40 late enhancer/promoter. In addition, promoters which are well known in the art which are induced in response to inducing agents such as metals, glucocorticoids, and the like, are also contemplated in the invention. Thus, it will be appreciated that the invention should be construed to include the use of any promoter/regulator sequence which is either know or is heretofore unknown, which is capable of driving expression of the nucleic acid operably linked thereto.

The invention also includes a method of obtaining a purified population of human HSCs. The method comprises two steps. The first step involves the purification of hematopoietic progenitor cells from cells obtained from human hematopoietic tissue. Such progenitor cells, or blasts, may be purified by various methods capitalizing on the difference(s) in a physical property (*e.g.*, the cell density), a biochemical/biological property (*e.g.*, the ability to take up a dye), and/or the

expression of various surface markers, using established procedures well-known in the art.

In one embodiment, $CD34^+$ HPCs were isolated using established procedures described herein, wherein the $CD34^+$ HPCs are obtained from embryonic fetal liver (FL), cord blood (CB), adult bone marrow (BM) and normal or mobilized peripheral blood (PB, MPB). The preferred method for purification of these cells is by use of the miniMACS Multisort CD34 isolation system (Miltenyi, Bergisch Gladbach, Germany). However, other methods known in the art for purification of hematopoietic progenitor cells, including $CD34^+$ cells, or methods to be developed, may also be used to practice the present invention.

Further, although CD34 marker was used to isolate HPCs, other early markers such as c-kit, CD38, Thy-1, and AC133, and the like, may also be used to isolate such cells.

In addition, $CD34^-$ cells which are also lin^- may also be used as the population of HPCs which are then processed according to the second step of the method. As disclosed herein in the examples below, $CD34^-lin^-KDR^+$ cells also comprise HSCs and these cells are able to engraft non-human animals just as $CD34^+KDR^+$ cells also engraft these animals. Thus, the $CD34^-lin^-$ cells also comprise a useful population enriched for undifferentiated cells from which long-term repopulating human hematopoietic cells may be isolated.

CD34+ versus CD34- cells and lin+ versus lin- cells may be separated from each other by, for example, fluorescence activated cell sorting as disclosed herein. However, the present invention should not be construed to be limited to this method of selecting cells on the basis of the expression of various cell surface markers. Rather, other methods well-known in the art for obtaining fractions of cell populations are also encompassed by the present invention.

In the second step, the human hematopoietic progenitor cells isolated previously are selected for the expression of KDR. In one embodiment, the HPCs were separated by cell sorting into $CD34^+KDR^+$ (KDR^{bright}), $CD34^+$ $KDR^{+/\pm}$ (KDR^{dim})

and $CD34^+KDR^-$ cells using anti-KDR monoclonal antibody (*i.e.*, the 260.4 clone available from Gesellschaft für Biologische Forschung, GBF, Braunschweig, Germany, or any other MoAb or molecule recognizing KDR^+ cells).

Other methods known in the art for separation of cell subsets or methods to be developed, may also be used to practice the present invention. The herein described purification of KDR^+ cells may be modified by using any other reagent or combination of reagents such as any MoAb or combination of MoAbs used together with any reagent (*e.g.*, MoAbs) which specifically bind KDR. Thus, the present invention should not be construed to be limited to using MoAb 260.4, or any other antibody, to isolate cells expressing KDR.

Further, as discussed previously elsewhere herein, other early markers besides CD34 may be used to select human long-term repopulating HSCs in conjunction with KDR. As an example, AC133 is expressed on immature hematopoietic progenitor cells and stem cells (Miraglia et al., 1997, Blood 90:5013-5021; Yin et al., 1997, Blood 90:5002-5012). AC133 MoAbs recognize 20-60% of $CD34^+$ cells including $CD34^{+bright}$, $CD38^{-/dim}$, $HLA\text{-}DR^-$, $CD90^+$ and $CD117^+$ cells. Thus, instead of using $CD34^+$ or $CD34^-$ cells expressing KDR $AC133^+$ or $AC133^-$ cells expressing KDR may be utilized. The invention described herein, therefore, includes all reagents when used together with any reagent recognizing KDR such as, but not limited to, other early markers including c-kit receptor, Thy-1, vascular endothelial growth factor receptor I, vascular endothelial growth factor receptor III, Tie1, Tek, basic fibroblast growth factor receptor, flt3 receptor, and AC133, as well as the selection of cells which are negative for late markers such as lin, and the like.

Receptor-type tyrosine kinases (RTKs) constitute a family of proteins involved in growth and developmental processes activating various cellular responses during embryogenesis and adult life. To further characterize $CD34^+$ that express KDR RT-PCR for detection of RTKs VEGFRI (flt1), VEGFRIII (flt4), Tie1 and Tek in these KDR^+ subsets was applied by using RT-PCR methodology previously described in detail (Ziegler et al., 1999, Blood 93:3355-3368). RT-PCR analysis provided evidence

that RTKs Flt1, Flt4, Tie1 and Tek were expressed at transcriptional level in small numbers of highly purified $CD34^+KDR^+$. Thus, $CD34^+$ cells expressing KDR may be further subdivided into subsets that express or not RTKs by using RTK specific antibodies or any other reagent recognizing RTKs. The invention thus includes all technologies/methodologies aimed to further subdivide the $CD34^+$ population that are KDR^+ by means of reagents recognizing the above mentioned RTKs, any other RTKs or any other cell surface structure expressed on KDR^+ populations.

Thus, the invention includes a method of isolating a KDR+ cell by selecting for cells expressing an antigen coexpressed with KDR on the surface of cells. Such antigens coexpressed with KDR include, for example, VEGFRI (flt1), VEGFRIII (flt4). Thus, KDR+ cells may be isolated by selecting for cells that express VEGFRI and/or VEGFRIII which are known to coexpress with KDR.

The purified human HSCs in the KDR^+ and KDR^- cell population are then assayed based upon their capacity for long term hematopoietic repopulation *in vitro* and *in vivo*. In parallel, the HPCs present in these two cell populations are assayed for their capacity for *in vitro* short term generation of a hematopoietic progeny. The long-term repopulation HSCs, defined according to the criteria described in the Examples section, are virtually exclusively contained within the $CD34^+KDR^+$ and $CD34^+KDR^{+/\pm}$ fractions. Conversely, unilineage and bilineage HPCs are almost exclusively contained within the $CD34^+KDR^-$ fraction. This method of purification of HSCs from CB, adult BM and PB or MPB yields a suitable number of HSCs for *in vitro* and *in vivo* clinical use. The most preferred sources of purified HSCs are post-natal hematopoietic tissues (*e.g.*, CB, adult BM, PB, and MPB). However, other hematopoietic tissue sources include, for example, embryonic hematopoietic tissue (*e.g.*, yolk sac and embryonic liver), fetal hematopoietic tissue (*e.g.*, fetal liver, fetal bone marrow, and fetal peripheral blood).

The invention further includes a population of cells and a cell obtained using the above-disclosed method.

The invention also includes further purifying the population of long-term repopulating HSCs by growing $CD34^+KDR^+$ or $CD34^+KDR^{+/\pm}$ cells in mini-bulk culture under starvation conditions as described elsewhere herein. Starvation resistant cells obtained following culture constitute approximately 10-25% of the initial number of cells placed in serum-free culture in the absence of any HGF treatment, except for VEGF addition. However, the resulting starvation-resistant cells comprise approximately ≥80-95% putative HSCs thereby being greatly enriched as a result of the starvation selection. The particular conditions for starvation culture are set forth elsewhere herein. One skilled in the art, based upon the disclosure provided herein, would appreciate that the particular conditions, *e.g.*, the precise number of days, may be varied so long as serum and HGFs are not added into the medium in any significant amount. The resultant starvation resistant cells, which are greatly enriched for *in vitro* long-term repopulating HSCs, may then be used in a wide variety of applications as described elsewhere herein. The invention includes a population of cells and a cell isolated by this method.

In addition, the invention includes a cell obtained by the above-disclosed method which cell comprises a nucleic acid. As described previously elsewhere herein, the nucleic acid may be operably linked to a promoter/regulatory sequence and/or may encode a variety of proteins and/or nucleic acids which are expressed by the cell and/or which inhibit expression of a nucleic acid complementary to the nucleic acid introduced into the cell.

The invention also includes a method of expanding human HSCs *in vitro* for use in either *ex vivo* or *in vivo* therapy. The method comprises obtaining a population of KDR^+ stem cells according to the above described method and incubating this cell population in the presence of VEGF. Further, the invention includes a population of cells and a cell obtained using this method.

In addition, the invention includes incubating KDR+ cells in the presence of VEGF and at least one other growth factor. As the data presented herein establish, treatment of the $CD34^+KDR^+$ cell population with VEGF results in a

significant increase in the number of HSCs. Addition of both VEGF and other suitable HGFs, as indicated herein, results in a marked amplification of the generated primitive HPCs, *i.e.*, approximately a 150-fold amplification of $CD34^+/CD38^+$ HPCs. In one aspect, the HGFs include, but are not limited to, flt3 receptor ligand, kit receptor ligand, thrombopoietin, basic fibroblast growth factor, interleukin 6, interleukin 3, interleukin 11, granulomonocytic colony-stimulatory factor, granulocytic colony-stimulatory factor, monocytic colony-stimulatory factor, erythropoietin, angiopoietin, and hepatocyte growth factor.

Purified HPCs may be differentiated for use in transfusion medicine. In this regard, a combined step procedure is applied to cells in culture. In one step, the purified $CD34^+KDR^+$ and/or the $CD34^-lin^-KDR^+$ population of long-term repopulating human HSCs is amplified which results in the generation of HSCs/HPCs by addition of VEGF and other suitable HGFs as described herein. In another step, the generated HSC/HPC population is grown in culture conditions which selectively channel the HPCs into differentiation and maturation through the erythroid or megakaryocytopoietic or granulopoietic/neutrophilic or monocytopoietic pathway (Labbaye et al., 1995, J. Clin. Invest. 95:2346-2358; Guerriero et al., 1995, Blood 86:3725-3736; Gabbianelli et al., 1995, Blood 86:1661-1670) or other hematopoietic pathways including granulopoietic/eosinophilic or basophilic, or dendritic cells, or B, or T lymphopoietic or NK cell pathways. Other methods known in the art for hematopoietic cell production or methods to be developed, may also be used.

Purified HSCs are also useful in a variety of clinical settings. For example, HSCs may be used as delivery vehicles for the administration of nucleic acid which is a therapeutic product or a nucleic acid encoding a therapeutic product (*i.e.*, an RNA or protein molecule) to a human. For example, HSCs are transfected/transduced with a suitable nucleic acid, preferably operably linked to a suitable promoter/regulatory sequence, wherein when the nucleic acid is expressed in the HSCs, a therapeutic RNA or protein is produced which is of benefit to the human. Delivery of

a nucleic acid to HSCs is accomplished using standard technology, for example, using viral gene transfer, described, for example, in Verma et al. (1997, Nature 389:239-242).

HSCs comprising an isolated nucleic acid may be readily introduced into the circulating blood by intravenous injection or infusion, intraperitoneal injection or infusion and even by intrauterine injection of infusion. Following delivery of HSCs to the circulating blood, they home to bone marrow microenvironmental niches.

Therapeutic nucleic acids which are suitable for introduction into HSCs include, but are not limited to, nucleic acid encoding adenosine deaminase or a biologically active fragment thereof, for treatment of severe combined immunodeficiency, the gene encoding β-globin, or a biologically active fragment thereof, for treatment of β-thalassemia or sickle cell anemia, a nucleic acid comprising an antisense HIV sequence, for example, an anti-tat nucleic acid sequence, for treatment of HIV infection, a nucleic acid encoding a multidrug resistance gene to facilitate drug resistance in transfected cells during treatment of neoplasia, and the like.

Suitable promoter/regulatory sequences include, but are not limited to, the retroviral LTR and the cytomegalovirus immediate early promoter.

The invention also includes a blood substitute comprising the progeny cells derived from an isolated purified population of long-term repopulating human hematopoietic stem cells as described in the experimental examples that follow.

In one aspect, the blood substitute comprises multi-oligo- and/or unipotent progenitors. In another aspect the blood substitute comprises red blood cells and/or neutrophilic granulocytes and/or eosinophilic granulocytes and/or basophilic granulocytes and/or monocytes and/or platelets, among other cells and/or components of normal blood. In another aspect the blood substitute comprises dendritic cells and/or T and/or B lymphocytes and/or NK cells. The physiological functions of the blood substitute described herein comprise the long-term repopulating HSC which permanently and completely reconstitutes the hematopoietic system of a myeloablated host, differentiated/ differentiating progeny generated from the cell(s) described previously elsewhere herein by *ex vivo* manipulation procedures yielding multi-, oligo-

and/or unipotent progenitors or terminal differentiated cells of the erythroid, granulocytic, monocytic, dendritic/antigen-presenting cells, megakaryocytic, T- and B-lymphoid, and natural killer (NK) cell series. These blood elements function as oxygen carriers (erythroid elements), phagocytes protecting the organism against infection (neutrophilic, eosinophilic, basophilic, granulocytes and monocytes/macrophages), producers (plasma cells/B-lymphocytes) of immunoglobulins (humoral immunity) which react with particular antigens, antigen-recognizing cells (T-cells; cell-mediated immunity), antigen-presenting cells (such as dendritic cells which process antigens intracellularly to peptides and present them together with MHC Class I or II molecules to CD8 and CD4 T-lymphocytes, respectively), cells killing other cells directly or by antibody-dependent cell-mediated cytotoxicity (ADCC) which they recognize as foreign (NK cells, lymphokine-activated killer cells, *i.e.*, LAK cells), producers of platelets (megakaryocytes) which play a central role in the haemostatic response to vascular injury.

The invention also includes a method of obtaining a purified population of long-term repopulating human hematopoietic stem cells that are $CD34^-lin^-KDR^+$, as these are defined by the examples set forth below. The method comprises obtaining a population of $CD34^-lin^-$ (lineage marker negative) cells and isolating a KDR^+ population therefrom. This is because, as more fully set forth below, $CD34^-lin^-KDR^+$ cells comprise another population comprising long-term repopulating human HSCs. Indeed, without wishing to be bound by theory, the $CD34^-lin^-$ KDR^+ cells may convert to their $CD34^+$ counterparts *in vivo* as $CD34^-lin^-$ cells convert into $CD34^+lin^-$ cells *in vitro* (Zanjani et al., 1998, Blood (Suppl. I) 92:504). Therefore, a purified population of long-term repopulating HSCs may be obtained by first selecting for $CD34^-lin^-$ by fluorescence activated cell sorting or by use of immunobeads as described elsewhere herein for isolation of $CD34^+$ cells and then further selecting from the $CD34^-lin^-$ population the subfraction of KDR^+ as described elsewhere herein. The use of antibodies specific for human cell markers to obtain purified populations of cells is well-known in the art and is described elsewhere herein. Other methods known in the

art for separation of cell subsets or methods to be developed, may also be used to practice the present invention, as discussed above for $CD34^+$ and $CD34^+KDR^+$ cell populations.

The long-term repopulating HSCs obtained by selecting for a population of $CD34^-lin^-$ KDR^+ cells may then be used similarly to the $CD34^+KDR^+$ previously described elsewhere herein such as, for example, as a blood substitute, for administration of a nucleic acid which is therapeutic, and/or in transplantation medicine.

The invention includes a chimeric mammal engrafted with at least one of an isolated purified long-term repopulating human hematopoietic stem cell. That is, the invention includes a mammal that has received an HSC from another mammal or an autologous transplant wherein the HSC is reintroduced into the mammal after being isolated and purified from that same mammal by *ex vivo* methods such as those described elsewhere herein. Thus, HSCs isolated from a mammal may be re-introduced into the same mammal or another mammal perhaps after a nucleic acid has been introduced into the cell. The present invention should be construed to encompass the introduction of a nucleic acid into a mammal by the process of introducing an isolated nucleic acid into an HSC removed from that animal and using the HSC to engraft the animal. The HSCs may be isolated from the same recipient animal or it may be obtained from another donor animal of the same or a different species. However, the invention should not be construed to be limited to only this method of producing a chimeric animal. Instead, the invention encompasses the production of a chimeric animal by other methods well known in the art such as, but not limited to, blastocyst injection. Such methods are well-known in the art.

The introduction of an isolated nucleic acid into an HSC has been described elsewhere herein and the methods for expanding the HSCs and for introducing them and thereby engrafting an animal with the cells are described elsewhere herein. One skilled in the art, based upon the disclosure provided herein, would be able to generate a chimeric mammal engrafted by at least one isolated

repopulating HSC by intravenous transfusion into the animal. However, any other method of delivering repopulating HSCs to mammal recipients may be used. Further, the recipient animal's hematolymphopoietic system may be either ablated before engraftment of the cell or the cell(s) are introduced into the animal in addition to the animal's own hematopoietic system.

Hematopoietic multilineage engraftment in the recipient mammal is defined as permanent and complete, *i.e.*, reconstitution of all hematopoietic lineages through donor HSCs, as well as sustained production of HPCs. Multilineage engraftment is detectable through specific MoAbs recognizing cells pertaining to a particular lineage. As an example, erythroid cells are recognized by anti-glycophorin A (GPA) MoAb, MKs are recognized by MoAbs such as anti-CD61 or -CD41, and HPCs are recognized by clonogenic assay and anti-$CD34^{+}$, anti-AC133 MoAbs, and the like.

The invention also includes a method of inhibiting rejection of a transplanted organ. The method comprises engrafting the organ recipient using an isolated and purified long-term repopulating human hematopoietic stem cell obtained from the organ donor prior to transplanting the organ. The bone marrow of the recipient is ablated by standard methods well known in the art. Generally, bone marrow ablation is accomplished by X-radiating the animal to be transplanted, administering drugs such as cyclophosphamide or by a combination of X-radiation and drug administration. In some embodiments, bone marrow ablation is produced by administration of radioisotopes known to kill metastatic bone cells such as, for example, radioactive strontium, ^{135}Samarium, or ^{166}Holmium (Applebaum et al., 1992, Blood 80:1608-1613). By engrafting the hematopoietic system of the recipient with HSCs from the organ donor, rejection of the transplanted organ is thereby inhibited.

Similarly, the invention includes a method of transplanting an autologous human hematopoietic stem cell in a human. The method comprises isolating a population of long-term repopulating stem cells from the recipient and ablating the bone marrow of the recipient. Non-malignant long-term repopulating stem

cells are then isolated by selecting for KDR^+ cells as disclosed previously elsewhere herein. Non-malignant cells are identified within a population of KDR^+ cells based on various criteria well-known in the art including, but not limited to, the cell morphology, biochemical properties, growth characteristics, and the expression of specific tumor cell markers. Thus, the bone marrow of the individual is purged of malignant blasts and other malignant cells such that by transplanting the non-malignant stem cells back into to the individual, diseases such as melanomas may be treated. That is, for diseases where the malignant cells do not express KDR, the bone marrow may be ablated and cells previously obtained from the individual may be enriched for non-malignant long-term repopulating hematopoietic stem cells and returned to the patient where they cause multi-lineage engraftment thereby treating or alleviating the disease.

The invention includes a method of isolating a KDR^+ stem cell giving rise to at least one of a skeletal muscle cell and a hepatic oval cell. The method comprises isolating a population of long-term repopulating HSCs by selecting for KDR^+ cells from cells obtained from human hematopoietic tissue as disclosed previously elsewhere herein. Recent data demonstrate stem cells associated with the bone marrow has epithelial cell lineage capability in that the cells gave rise to repopulating liver cells in transplanted rats (Petersen et al., 1999, Science 284:1168-1170). Similarly, Ferrari et al. (1998, Science 279:1528-1530), demonstrated that unfractionated bone marrow cells, when injected into recipient muscle, migrated to sites of muscle damage, and gave rise to marrow-derived cells which underwent myogenic differentiation and participated in regeneration of damaged muscle fibers. Further, bone marrow cells have the potential to differentiate to lineages of mesenchymal tissues, including bone, cartilage, fate, tendon, muscle and marrow stroma (Pittenger et al., 1999, Science 284:143-147). Thus, mesenchymal, hepatic and myogenic progenitors may be recruited from marrow-derived cells. Without wishing to be bound by theory, the stem cells which gave rise to hepatic oval cells and myogenic progenitors are likely to be the long-term repopulating KDR^+ stem cells of

the present invention. Thus, by isolating KDR+ stem cells as disclosed herein, it is possible to derive cells with epithelial cell and/or myogenic capability.

The invention includes a method of monitoring the presence of KDR^+ stem cells in a human hematopoietic tissue in a human receiving therapy. The method comprises obtaining a hematopoietic tissue sample from the human and measuring the number of KDR+ stem cells in the sample. Measurements are made before, during and after therapy where therapy may be chemotherapy and/or radiation therapy which is known to affect the stem cell compartment such as, for example, myeloablation therapy or therapy known to cause hematopoietic suppression. Until the present invention, no method was available to allow the status of the stem cell compartment to be determined during such therapy. The present invention, by defining a marker, *i.e.*, KDR^+, for the cells of this compartment, allows the determination of the status of the stem cell compartment in a patient receiving therapy known or thought to affect the stem cell compartment at any point before, during, and after therapy.

Definitions

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "antibody", as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope on an antigen.

Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins.

Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies and humanized antibodies (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Harlow et

al., 1988, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al.,1988, Proc. Natl. Acad. Sci. U.S.A. 85:5879-5883; Bird et al., 1988, Science 242:423-426). By the term "specifically binds", as used herein, is meant, for example, an antibody which recognizes and binds CD34 polypeptide, but does not substantially recognize or bind other molecules in a sample. Similarly, an antibody "specifically binds KDR" if the antibody recognizes and binds VEGFR2/KDR/flk-1 in a sample but does not substantially recognize or bind to other molecules in a sample. Further, an antibody specifically binds lin markers if the antibody recognizes and binds lineage markers but does not substantially recognize or bind to other molecules in a sample.

By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

As used herein, the term "antisense nucleic acid" means a nucleic acid polymer, at least a portion of which is complementary to another nucleic acid. The antisense nucleic acid may comprise between about fourteen and about fifty or more nucleotides. Preferably, the antisense nucleic acid comprises between about twelve and about thirty nucleotides. More preferably, the antisense nucleic acid comprises between about sixteen and about twenty-one nucleotides. The antisense nucleic acid may include, but is not limited to, phosphorothioate oligonucleotides and other modifications of oligonucleotides. Methods for synthesizing oligonucleotides, phosphorothioate oligonucleotides, and otherwise modified oligonucleotides are well known in the art (U.S. Patent No: 5,034,506; Nielson et al., 1991, Science 254:1497).

The term "antisense" refers particularly to the nucleic acid sequence of the non-coding strand of a double stranded DNA molecule or, in the case of some viruses, a single or double stranded RNA molecule, encoding a protein, or to a sequence which is substantially homologous to the non-coding strand. It is not necessary that the antisense sequence be complementary solely to the coding portion of the coding strand of the nucleic acid molecule. The antisense sequence may be complementary to regulatory sequences specified on the coding strand of a nucleic acid molecule encoding a protein, which regulatory sequences control expression of the coding sequences.

The term "sense", as used herein, refers to the nucleic acid sequence of the single or double-stranded nucleic acid molecule which encodes a protein, or a sequence which is substantially homologous to that strand. However, the nucleic acid sequence is not limited solely to the portion of the coding strand encoding a protein; rather, the sequence may include regulatory sequences involves in, for example, the control of expression of the coding sequence.

The term "biochemical/biological property," as used herein, means any biochemical/biological property of a cell which allows the purification of such cell. A biochemical/biological property includes, for example, the ability of a cell to take up or exclude certain dyes.

"Blood substitute," as used herein, refers to a substance derived from long-term repopulating human hematopoietic stem cells comprising at least one component of naturally-occurring blood such as, for example, red blood cells, platelets, and other components/products of normal blood. Further, the blood substitute refers to a substance that can perform at least one of the biochemical/physiological functions of normal blood such as the transport of oxygen, and the like.

By "chimeric mammal" as the term is used herein, is meant any mammal which is a recipient of at least one long-term repopulating human HSC from another mammal.

"Complementary" as used herein refers to the broad concept of subunit sequence complementary between two nucleic acids, *e.g.*, two DNA molecules. When a nucleotide position in both of the molecules is occupied by nucleotides normally capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are complementary to each other when a substantial number (at least 50%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (*e.g.*, A:T and G:C nucleotide pairs).

By the terms "coding" and "encoding", as these terms are used herein, is meant that the nucleotide sequence of a nucleic acid is capable of specifying a particular polypeptide of interest. That is, the nucleic acid may be transcribed and/or translated to produce the polypeptide. Thus, for example, a nucleic acid encoding adenosine deamininase is capable of being transcribed and/or translated to produce an adenosine deamininase polypeptide.

"Coexpressed," as the term is used herein, means that the antigen is expressed on or in a cell which also comprises detectable KDR antigen. However, the two molecules need not be coexpressed contemporaneously. Rather, it is sufficient that the cell express both KDR and the coexpressed antigen at some point in time such that selection of a cell expressing the other antigen selects for cells which either at that moment, or at some later time, also express KDR.

The term "early marker," as used herein, means any antigen on the surface of a cell which is preferentially or selectively expressed on the surface of undifferentiated precursor cells compared to its expression on the surface of differentiated cells. Examples of early markers for hematopoietic cells include, but are not limited to, CD34, Thy-1, c-kit receptor, flt3 receptor, AC133, vascular endothelial growth factor receptor I, vascular endothelial growth factor receptor III, Tie1, Tek, and basic fibroblast growth factor receptor.

By "engrafted", as the term is used herein, is meant that the mammal comprises a hematolymphopoietic system repopulated by multi-lineage cells derived from at least one isolated purified HSC which was administered to the animal.

The term "enriched," as used herein, means that a population of cells comprises a detectably higher level of the enriched cell type than an otherwise identical cell population not subjected to selection for that cell type. The level of enrichment may be determined by comparing the number of cells of interest in an unselected population to the number of cells of interest in a population selected for a particular trait or marker by a cell selection method.

An "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, *e.g.*, a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, *e.g.*, RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

The term "KDR^{+}," as used herein, means the cell expresses detectable KDR antigen. The antigen may be detected by a variety of methods including PCR, RT-PCR, Western blotting, and immunofluorescence. With regard to immunofluorescence, KDR^{+} cells may be designated KDR+ (*i.e.*, KDR^{bright}) and $KDR^{+/\pm}$ (*i.e.*, KDR^{dim}) when stained using the anti-KDR monoclonal antibody 260.4 under the conditions disclosed elsewhere herein.

By the term "late marker," as used herein, is meant a marker associated with or preferentially expressed on differentiated precursor cells. Such markers include, but are not limited to, the lineage (lin) markers.

By the term "multi-lineage engrafting dose", as the term is used herein, is meant at least one long-term repopulating human hematopoietic stem cell which, when transplanted into an animal, is capable of giving rise to detectable multi-lineage engraftment of the recipient animal.

"Non-malignant," as the term is used herein, means that a cell does not exhibit any detectable traits typically associated with neoplastic cells such as the loss of contact-inhibition, and the like.

The term "physical property," as used herein, means any property of a cell which may be used to physically isolate such cell. For example, physical properties of a cell include, but are not limited to, the cell size, density, mass, and morphology.

As used herein, the term "promoter/regulatory sequence" means a DNA sequence which is required for expression of a gene operably linked to the promoter/regulator sequence. In some instance, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene in a tissue-specific manner.

By describing two nucleic acid sequences as "operably linked" as used herein is meant that a single-stranded or double-stranded nucleic acid moiety comprises each of the two nucleic acid sequences and that the two sequences are arranged within the nucleic acid moiety in such a manner that at least one of the two nucleic acid sequences is able to exert a physiological effect by which it is characterized upon the other.

By "starvation resistant," as the term is used herein, is meant that a cell has the ability to survive at least about 5-10 days (shorter starvation times may apply) in liquid suspension culture in FCS-free and serum-free medium (or any other type of

suitable medium) in absence of added HGFs, except VEGF, under the conditions described elsewhere herein.

"Transfected" or "Transduced", as the term is used herein, encompasses any method by which an isolated nucleic acid may be introduced into a cell. Such methods are well known in the art and are described in, for example, Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York) and Ausubel et al. (1997, Current Protocols in Molecular Biology, Green & Wiley, New York). For instance, the nucleic acid may be introduced into a cell using a plasmid or viral vector, electroporation, a "gene gun", polylysine compounds, and the like.

By the term "vector" as used herein, is meant any plasmid or virus encoding an exogenous nucleic acid. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into virions or cells, such as, for example, polylysine compounds and the like. The vector may be a viral vector which is suitable as a delivery vehicle for delivery of the isolated nucleic acid of interest (*e.g.*, adenosine deamininase, β-globin, multidrug resistance, and the like) to a cell, or the vector may be a non-viral vectors which is suitable for the same purpose. Examples of viral and non-viral vectors for delivery of nucleic acids to cells and tissues are well known in the art and are described, for example, in Ma et al. (1997, Proc. Natl. Acad. Sci. U.S.A. 94:12744-12746). Examples of viral vectors include, but are not limited to, a recombinant vaccinia virus, a recombinant adenovirus, a recombinant retrovirus, a recombinant adeno-associated virus, a recombinant avian pox virus, and the like (Cranage et al., 1986, EMBO J. 5:3057-3063; International Patent Application No. WO94/17810, published August 18, 1994; International Patent Application No. WO94/23744, published October 27, 1994). Examples of non-viral vectors include, but are not limited to, liposomes, polyamine derivatives of DNA, and the like.

The invention will be further described by reference to the following experimental examples. These examples are provided for purposes of illustration

only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any all variations which become evident as a result of the teaching provided herein.

Examples

The experiments which are presented herein examine the expression and functional role of VEGFR, particularly the VEGFRII termed flk1/KDR, in HPCs/HSCs purified from embryonic-fetal liver (FL), cord blood (CB), normal or mobilized adult peripheral blood (PB, MPB) and adult bone marrow (BM). As indicated herein, these purified lin^- (lineage marker negative) HPC populations comprise a small minority of HSCs. The data may be summarized as follows.

KDR expression on purified $CD34^+$ HPC populations was analyzed using a monoclonal antibody (MoAb) which recognizes the extracellular receptor domain.

MoAb evaluation indicated that KDR is expressed on approximately $<1\%$ CB, BM, PB or MPB $CD34^+$ cells under the conditions used herein. Representative results using this MoAb indicated that KDR is expressed on approximately $\geq 1\%$ FL $CD34^+$ cells. Without wishing to be bound by theory, other antibodies and/or varying detection conditions may affect the percentage of KDR+ cells detected in a CD34+ population of cells.

KDR expression is virtually restricted to adult and CB HSCs and a portion of the most primitive subset of adult and CB HPCs. KDR is also expressed on approximately $\leq 1\%$ of $CD34^-lin^-$ cells.

The KDR^+ versus KDR^- cell fractions were sorted from $CD34^+$ HPCs purified from CB, BM, PB or MPB. In both cell fractions, the following assays were performed: (i) Assay of HPCs in clonogenic culture; (ii) assay of long-term repopulating HSCs *in vitro* (*i.e.*, evaluation in 12 week LTC of the frequency of CAFCs and/or LTC-ICs: the frequency was evaluated by limiting dilution assay (LDA)) and *in vivo*, *i.e.*, analysis of hematopoietic repopulation in

NOD-SCID mice at 3 months after sublethal irradiation and cell injection. The results consistently established that the $CD34^+KDR^+$ and/or the $CD34^+KDR^{+/\pm}$fraction contained little or no uni-oligopotent HPCs, and a minority of multipotent and primitive HPCs, whereas it was dramatically enriched for HSCs. Conversely, the $CD34^+KDR^-$ fraction contained virtually all uni-oligopotent HPCs, as well as multilineage and primitive HPCs, and essentially no long-term repopulating HSCs.

In clonogenic semisolid culture, treatment of $CD34^+KDR^+$ cells with VEGF, combined with diverse cocktails of hematopoietic growth factors (HGFs), caused a mild stimulatory effect on multipotent HPCs and primitive HPCs. More importantly, LDA of LTC-IC/CAFC frequency in the KDR^+ and KDR^- cell fraction from PB, BM, or CB in Dexter type 12 week LTC revealed that, in PB, BM and CB KDR^+ cell fractions, the LTC-IC/CAFC frequency was elevated (approximately ≥50-60%, representative results) in LTC supplemented with VEGF, whereas it was lower (approximately 25-43%, representative results) in PB, BM and CB LTC which were not supplemented with VEGF. In both BM and CB KDR^- cell fractions, the LTC-IC/CAFC frequency was 0% or close to 0% with or without VEGF treatment. Similar results on LTC-ICs/CAFCs were obtained in MPB KDR^+ cells. In preliminary experiments, twelve week incubation of normal PB KDR^+ cells with VEGF in single cell LTC, followed by seeding the generated cells into secondary LTC, caused an amplification of the number of HSCs, assayed as 12 week LTC-ICs. In addition, liquid suspension culture experiments on $CD34^+KDR^+$ vs $CD34^-KDR^-$ CB cells confirmed that only the KDR^+ cell fraction generated in the long-term (approximately 12 week culture) primitive $CD34^+CD38^-$ HPCs, particularly when stimulated by not only early acting HGFs (see below) but by VEGF combined with early acting HGFs. $CD34^+KDR^+$ cells seeded in single cell or minibulk FCS^- free HGF^- starvation culture partially survived for up to at least 1 month upon addition of VEGF. The starvation resistant cells were enriched for putative HSCs (up to approximately ≤80-95%, representative results).

These data therefore establish the following. VEGFRII (KDR) expression is restricted to a small subset of CB, BM, PB and MPB $CD34^+$ HPCs. This subset comprises virtually no uni- or oligopotent HPCs, a fraction of primitive HPCs and virtually the entire pool of long-term repopulating $CD34^+$ HSCs, respectively endowed with modest or extensive self-renewal capacity. Consistent with these results, VEGF selectively stimulates the proliferation of and/or protects against apoptosis primitive HPCs and particularly HSCs.

Furthermore, preliminary experiments suggest that KDR^+ cells in the $CD34^-lin^-$ cell population purified from adult hematopoietic tissues also contained a fraction of long-term repopulating HSCs. Therefore, the data disclosed herein demonstrate that KDR is novel key marker for human long-term repopulating HSCs and that the VEGF/KDR system plays a key role in long-term HSC function.

The Materials and Methods used in the experiments presented herein are now described.

VEGFRII (KDR) antibody

The mouse monoclonal antibody (clone 260.4), raised against the KDR soluble protein and recognizing the extracellular KDR domain were obtained from Gesellschaft für Biologische Forschung, GBF, Braunschweig, Germany.

Hematopoietic growth factors (HGFs)

Recombinant human HGFs were purchased from commercial sources (see below); VEGF was purchased from R&D Systems (Minneapolis, MN).

Cells and purification procedures

Human HPCs (containing a small HSC subpopulation), and the KDR^+ fraction thereof, were purified from (i) fetal liver (FL), (ii) cord blood (CB), (iii) adult bone marrow (BM), and (iv) adult normal or mobilized peripheral blood (PB, MPB), as described below.

$CD34^+$ cell purification

BM cells were obtained from consenting normal donors. MPB was obtained from G-CSF-treated (5 μg/kg/day) consenting normal donors. Normal PB

was collected as buffy coat preparation from the local blood bank. CB was obtained from healthy, full-term placentas according to institutional guidelines. Low-density cells (<1.077 g/ml) were isolated by Ficoll and $CD34^+$ cells purified by MiniMACS column (Miltenyi Bergisch Gladbach, Germany and Auburn, CA).

Fluorescence staining and flow cytometry analysis

Purified $CD34^+$ cells were incubated for 30 min on ice with saturating amounts of biotinylated anti-KDR MoAb (clone 260.4, Gesellschaft für Biologische Forschung, Braunschweig, Germany) and anti-CD34 FITC MoAbs (clone HPCA-2, Becton-Dickinson (B-D), San Jose, CA). For three color FACS analysis, anti-CD34 PerCP and one of following FITC-conjugated MoAbs were used: anti-CD38 (B-D), anti-flt3 (Immunotech, Marseille, France), anti-Thy-1 (Pharmingen, San Diego, CA), anti-c-kit (Serotec, Oxford, UK). The cells were then washed and labeled with streptavidin-PE (B-D). After a further washing, cells were run on a FACScan or FACSCalibur for two- or three-color analysis.

$CD34^+KDR^+$ cell separation

Purified $CD34^+$ cells were incubated with saturating amounts of anti-CD34-FITC and biotinylated anti-KDR, washed and labeled with streptavidin-PE (B-D). After a further washing, $CD34^+KDR^+$ or $KDR^{+/\pm}$ and $CD34^+KDR^-$ subpopulations were sorted on FACSVantage (B-D) or EPICS Elite (Coulter) (fluorescence emission, 525 and 575 nm). A fraction of sorted KDR^- cells was reanalyzed: if contaminating KDR^+ cells were detected, the population was restained and resorted to ensure elimination of all KDR^+ cells.

KDR RT-PCR was performed as described (Ziegler et al., 1999, Blood 93:3355-3368) using 5'-AAAACCTTTTGTTGCTTTTTGA-3' [SEQ ID NO:1] and 5'-GAAATGGGATTGGTAAGGATGA-3' [SEQ ID NO:2] primers (Terman et al., 1991, Oncogene 6:1677-1683).

In vitro assays.

HPC assay.

HPCs were seeded in 0.9% methylcellulose fetal calf serum free (FCS^-) medium supplemented with saturating amounts of HGFs [flt3, kit ligand (FL, KL), basic fibroblast GF (bFGF) (100 ng/ml each), interleukin 6 (10 ng), IL3 (100 U), granulomonocyte colony-stimulating factor (GM-CSF) (10 ng), G-CSF (500 U), M-CSF (250 U), thrombopoietin (Tpo) (100 ng), erythropoietin (Epo) (3 U)]. CFU-Mix/BFU-E and CFU-GM colonies comprised >5 x 103 and $>10^3$ cells, respectively (Gabbianelli et al., 1995, Blood 86:1661-1670). A more limited HGFs combination comprised IL3, GM-CSF, Epo at the indicated dosages (Gabbianelli et al., 1995, Blood 86:1661-1670) (this culture condition was also utilized for NOD-SCID mice BM mononuclear cell (MC) clonogenic assay). CFU-Mix/BFU-E and CFU-GM colonies comprised >500 and >100 cells respectively. For detection of human colonies, the colony DNA was processed for PCR using KlenTaq-1 DNA polymerase (Clontech, Palo Alto, CA) and primers recognizing human a-satellite sequences on chromosome 17 (Warburton et al., 1991, Genomics 11:324-333).

HPP-CFC assay

HPP-CFC assay ws performed as described in Gabbianelli et al., (1995, Blood 86:1661-1670). Primary HPP-CFC clones, scored at day 30, were replated for secondary HPP-CFC colony formation.

Five-8-12-wk LTC.

The LTC were established on allogeneic irradiated (20 Gy) BM stromas (Gabbianelli et al., 1995, Blood 86:1661-1670) or FBMD-1 cells (van der Loo et al., 1995, Blood 85:2598-2606). At weekly intervals half of the medium was removed and replaced by fresh medium $\pm$ VEGF (100 ng/ml). In 12-wk LTC irradiated BM stromas or fresh FBMD-1 cells were added monthly to prevent functional exhaustion of the initial inoculum (Hao et al., 1996, Blood 88:3306-3313). In minibulk LTC each well was seeded with 100-1,000 $CD34^+KDR^+$ cells (1,000 cells/ml) (positive or negative control was seeded with 10,000 $CD34^+$ or $CD34^+KDR^-$ cells respectively). LTC were terminated at 5-8-12-wk: cells from supernatant and adherent fractions were cultured in semisolid medium for colony

growth (Gabbianelli et al., 1995, Blood 86:1661-1670). Alternatively, 6-9-12-wk CAFCs were scored directly in LTC adherent layer (van der Loo et al., 1995, Blood 85: 2598-2606).

Limiting dilution assay (LDA)

Graded numbers of $CD34^+KDR^+$ cells (1-100 cells/well) were seeded in LTC wells (Sutherland et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:3584-3588; Carè et al., 1999, Oncogene 18:1993-2001). The frequency of 12-week LTC-ICs/CAFCs was calculated according to single hit Poisson statistics (Sutherland et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:3584-3588; Carè et al., 1999, Oncogene 18:1993-2001). Control LDA was performed on $CD34^+KDR^-$ cells (10-5,000 cells/well) and unseparated $CD34^+$ cells (20-5,000 cells/well).

Liquid phase suspension culture

Liquid phase suspension culture in FCS^- medium ± VEGF and ± other HGFs was performed as in described in Ziegler et al. (1999, Blood 93:3355-3368). In the representative minibulk (2-3 x 10^3 $CD34^+KDR^{+/\pm}$ or $CD34^+KDR^-$ cells/well) or in single cell (1 $CD34^+KDR^{+/\pm}$ or $CD34^+KDR^-$ cell/well) starvation culture experiments, cells were treated only with VEGF (100 ng/ml). In a VEGF ± HGFs representative experiment 1,000 purified $CD34^+$ KDR^+ or $CD34^+KDR^-$ CB cells were grown in 100 μl of FCS-free medium (Gabbianelli et al., 1995, Blood 86:1661-1670) in individual wells of a 96-well plate until cell numbers reached approximately 10,000/well on or about day 14. Thereafter, the cells were transferred to individual wells of a 24-well plate with 500μl of medium. Cultures were supplemented with VEGF (50 ng/ml) either alone or combined with Tpo (100 ng/ml), FL (100 ng/ml), IL-3 (0.1 ng/ml). HGF combinations were VEGF alone, VEGF+FL, VEGF+FL+Tpo, VEGF+FL+IL-3, and FL+Tpo+IL-3. At weekly intervals, one half of the medium was replaced by fresh medium and HGFs. Starting at day 25 of culture, cell numbers were determined weekly and immunophenotype analysis of cultured cells was performed

weekly using anti-CD34 and anti-CD38 MoAbs. The cultures were maintained for 12 weeks.

NOD-SCID mice xenografts

Six-8-week old mice (Jackson Laboratory, Bar Harbor, ME) were irradiated at 3.5 Gy using a ^{137}Cs source (Gammacell) 12-24 hours prior to xenotransplantation. KDR^+ or KDR^- cells were injected by tail vein injection together with 100,000 irradiated (20 Gy) BM or CB mononuclear cells (MCs). Mice were killed 12 weeks after xenotransplantation according to institutional regulations. Cell suspensions from femurs, spleen and PB were analyzed for human cells by flow cytometry: erythrocytes depleted cells were labeled with FITC- or PE-conjugated MoAbs which specifically bound the following markers: CD45 (HLe1), CD34 (HPCA-2), CD38, CD15, CD33, CD71, CD2, CD3, CD4, CD7, CD8, CD19, CD20, CD16, CD56 (B-D); GPA, CD71 (Pharmingen, San Diego, CA). FITC- or PE-conjugated isotype-matched irrelevant MoAbs were used as controls. Bone marrow, spleen and PB cells from non-transplanted mice were used as negative control. Positive controls consisted of human BM or CB MCs. BMMCs were also cultured in semisolid media selective for human HPCs as described previously elsewhere herein.

Fetal sheep xenografts

Fetal sheep xenographs were performed as described previously (Zanjani et al., 1998, Exp. Hematol. 26:353-360; Civin et al., 1996, Blood 88:4102-4109; Kawashima et al., 1996, Blood 87:4136-4142; Sutherland et al., 1996, Exp. Hematol. 24:795-806; Uchida et al., 1996, Blood 88:1297-1305). PB and BM MCs from chimeric fetuses/newborns, separated by Ficoll gradient, were evaluated for presence of human cells by flow cytometry. BMMCs were also assayed for human HPCs in clonogenic culture by karyotyping of hematopoietic colonies. Human $CD34^+$ cells, isolated by MiniMACS column from BMMCs of primary recipients as described previously elsewhere herein, were transplanted in secondary recipients.

Receptor-type tyrosine kinases (RTKs) RT-PCR assay in $CD34^+KDR^+$ cells

BM $CD34^+KDR^+$ cells were isolated by double sorting and analyzed by RT-PCR (Ziegler et al., 1999, Blood 93:3355-3368). The following primers (Klagsbrun et al., 1996, Cytokine Growth Factor Rev. 7:259-270) were used for RT-PCR: VEGFRI/Flt1, 5'-AAACCAAGACTAGATAGCGTCA-3' [SEQ ID NO:3]; 5'-TTCTCACATAATCGGGGTTCTT-3' [SEQ ID NO:4]; VEGFRII/Flt4, 5'-GACAAGGAGTGTGACCACTGAA-3' [SEQ ID NO:5], 5'-TGAAGGGACATTGTGTGAGAAG-3' [SEQ ID NO:6]. The following primers (Sato et al., 1995, Nature 376:70-74), were also used: Tie1, 5'-GAGTCCTTCT TTGGGAGATAGTGA-3' [SEQ ID NO:7], 5'-GTCAGACTGGTCACAGGTTAGACA-3' [SEQ ID NO:8]; Tek, 5'-CATTTTTGCAGAGAACAACATAGG-3' [SEQ ID NO:9], 5'-TCAAG CACTGGATAAA TTGTAGGA-3' [SEQ ID NO:10].

$CD34^-lin^-$ cell purification

Purification of $CD34^-lin^-$ cells was performed as indicated in Bathia et al. (Nature Med. 4:1038-1045). The KDR^+ cell subfraction of the $CD34^-lin^-$ cell fraction was obtained as indicated previously elsewhere herein for $CD34^+$ cells.

The results of the Experiments presented herein are now described.

In preliminary studies, PB HPCs were purified and grown in unilineage differentiation cultures (Gabbianelli et al., 1990, Science 249:1561-1564; Testa et al., 1996, Blood 88:3391-3406). In accord with previous studies (Katoh et al., 1995, Cancer Res. 55:5687-5692), RT-PCR analysis confirmed that KDR mRNA was expressed in HPCs, but was not detected in the HPC progeny except for expression on megakaryocytes. Thereafter, a high-affinity monoclonal antibody (MoAb) which specifically binds the extracellular KDR domain was used to monitor KDR expression on HPCs from bone marrow (BM), normal peripheral blood (PB), mobilized peripheral blood (MPB), and cord blood (CB). Extensive FACS analysis on ≥ 98% purified $CD34^+$cell populations from these tissues indicated that KDR^+ cells represent a

minuscule subset of all $CD34^+$ cells, usually comprised in the <1% range (Figure 1A, top panel) as confirmed by RT-PCR analysis (Figure 1A, bottom right panel). A $KDR^{\pm}$ (KDR^{dim}) cell population has also been identified in $CD34^+$ cells (Figure 1A, bottom left panel) and occasionally cosorted with the KDR^+ (KDR^{bright}) fraction. BM, PB, MPB, and CB $CD34^+KDR^+$ cells, essentially lin^- (approximately <5-20% $CD45RA^+$, $CD13^+$, $CD33^+$, $CD61^+$, $CD19^+$ in representative experiments), are variably positive for early HPC/HSC markers (Figure 1B).

The hematolymphopoietic hierarchy is defined by functional assays. Pluripotent HSCs, endowed with extensive self-renewal capacity, are assayed *in vivo* on the basis of their capacity to repopulate the hematolymphopoietic system, *i.e.*, to xenograft irradiated NOD-SCID mice (Bhatia et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:5320-53-25; Wang et al., 1997, Blood 89:3919-3924; Conneally et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:9836-9841) and pre-immune fetal sheep (Zanjani et al., 1998, Exp. Hematol. 26:353-360). HSCs feed into primitive HPCs endowed with limited self-renewal potential but extensive proliferative capacity, which are identified *in vitro* as high proliferative potential colony-forming cells, HPP-CFCs (Brandt et al., 1990, J. Clin. Invest. 86:932-941) and more advanced lineage(s)-committed HPCs with no self-renewal activity (defined *in vitro* as colony- or burst-forming units, CFUs, BFUs) (Ogawa, 1993, Blood 81:2844-2853).

The 5-8 week LTC identifies LTC initiating cells (LTC-ICs), which represent primitive HPCs apparently distinct from *in vivo* repopulating HSCs (Larochelle et al., 1996, Nature Med. 2:1329-1337). The 12 week extended LTC identifies more primitive LTC-ICs, which are resistant to retroviral gene transfer (Hao et al., 1996, Blood 88:3306-3313), as repopulating HSCs (Larochelle et al., supra), and represent putative HSCs. Similarly, the LTC identifies 5 week (van der Loo and Ploemacher, 1995, Blood 85:2598-2606) and 12 week cobblestone area forming cells (CAFCs). The data disclosed herein, utilizing the HSC/HPC functional assays, demonstrate that in post-natal hematopoietic tissues, KDR represents a specific functional HSC marker, which is virtually not expressed on oligo-, uni-potent HPCs.

In vitro HPC/HSC assays

$CD34^+KDR^+$ cells were tested by *in vitro* HPC/HSC assays. Preliminary studies indicated that VEGF addition in $CD34^+$ cell culture exerts a mild stimulatory effect on multipotent CFU (CFU-Mix), HPP-CFCs and 8-wk LTC-ICs. Thereafter, $CD34^+$ cells were purified and the $CD34^+KDR^+$ or $CD34^+KDR^{+/\pm}$ subfractions were separated from the $CD34^-KDR^-$ subfraction (Figure 1A, bottom left). Both subsets were then assayed for HPCs, HPP-CFCs and 6,9 and 12 week CAFCs or 5, 8, and 12 week LTC-ICs.

HPC assay

In representative PB experiments, the addition of saturating levels of interleukin 3 (IL3), granulomonocytic colony-stimulatory factor (GM-CSF) and erythropoietin (Epo) demonstrated that oligo-unipotent HPCs (BFU-E, CFU-GM) were essentially restricted to the KDR^- cell fraction (Figure 2A, top left). Addition of a larger spectrum of HGFs, *i.e.*, including also early-acting HGFs c-kit ligand (KL), flt3 ligand (FL), IL6) as well as unilineage HGFs (thrombopoietin (Tpo), G-CSF, M-CSF), confirmed that virtually all oligo-unipotent HPCs are present in the KDR^- fraction (Figure 2A, top right). VEGF addition to the HGF cocktail did not modify this pattern, except for borderline increase of CFU-Mix in KDR^+ culture. Essentially similar results were obtained for CB, MPB and PB.

HPP-CFC assay

HPP-CFCs scored in primary and secondary cultures (*i.e.*, HPP-CFCs I and II, respectively) were present in both KDR^+ and KDR^- fractions. The frequency of HPP-CFC II was more elevated in the KDR^+ fraction (<10%) as compared to the KDR^- (<5%) population (Figure 1B, bottom panel depicts PB results). Again, VEGF addition did not significantly modify this pattern, except for a slight increase of HPP-CFC number in the KDR^+ cell culture. Similar results were obtained for BM and CB.

LTC-IC/CAFC assay

LTC-IC assay was performed in 5-, 8- and 12-week Dexter-type LTCs for $CD34^+$, $CD34^+KDR^{+/\pm}$, and $CD34^+KDR^-$ cells from BM, MPB, PB, and CB (*see, e.g.*, Figure 1C). The data disclosed demonstrate (Figure 1C, left panel) that in LTC seeded with PB $CD34^+$ cells, the number of HPC generated declined sharply from 5 through 12 weeks, but a small residual number of HPCs was still detected at 12 weeks. In $CD34^+KDR^-$ LTC, a similar decline was observed, but no residual HPCs were detected at 12 weeks. Notably, $CD34^+$KDR+ LTC exhibited a moderately low number of HPCs at 5 and 8 weeks, followed by a sharp increase of HPC generation at 12 weeks. An equivalent pattern was observed in BM, MPB and CB LTC, as evaluated in 6, 9, and 12 week CAFC assay (Figure 1C, middle and right panels).

Altogether, oligo-unipotent HPCs are essentially restricted to KDR^- cells, while putative HSCs (12 week CAFCs/ LTC-ICs) are restricted to KDR^+ cells. The intermediate primitive HPC populations (HPP-CFCs, 6-9 week CAFCs, 5-8 week LTC-ICs) are present in both cell fractions.

NOD-SCID mouse assays

Irradiated NOD-SCID mice were transplanted with $CD34^+$ (50,0000 to 250,000 cells/mouse), $CD34^+KDR^+$ or $CD34^+KDR^{+/\pm}$ (150 to 10,000 cells/mouse), or $CD34^+KDR^-$ (10,000 to 250,000 cells/mouse) from BM, CB, MPB or PB. In some experiments, $CD34^-lin^-$ KDR^+ cells were also injected. Mice recipients were sacrificed at 12 weeks post-transplant and cell suspensions were obtained from BM, spleen and PB of mouse recipients and were analyzed by FACS for the presence of human cells as described elsewhere herein. Consistent engraftment was observed using $CD34^+KDR^+$ cells and essentially no engraftment was observed using double sorted $CD34^+KDR^-$ donor cells.

NOD-SCID bone marrow studies

In a representative experiment (Figure 3A), between about 100 to about 1,600 $CD34^+KDR^+$ cells were injected into each NOD-SCID mouse recipient. In the negative control group, 250,000 double sorted $CD34^+KDR^-$ cells did not engraft, whereas unseparated CD34 cells demonstrated multilineage engraftment (Figure 3A,

top left panel). $CD34^+KDR^+$ cells always engrafted the recipient mouse. Moreover, the engraftment observed involved all hematopoietic lineages (*i.e.*, double labeling for $CD33^+15^+$ or $CD14^+45^+$ cells, $CD71^+GPA^+$ cells and $CD45^+41^+$ cells, pertaining to granulomonocytic, erythroid and megakaryocytic series, respectively) in representative mice (Figure 3A, bottom panel). Further, the engraftment involved both B and T lymphoid compartments (*i.e.*, $CD19^+20^+$ and $CD4^+8^+3^+$ cells, respectively), as well as NK cells ($CD16^+56^+$ cells) (Figure 3A). A dose-response was observed from 100 through 1,600 cells for all engrafted cell populations (Figure 3A, top), particularly for $CD45^+$ cells (Figure 3A, top). Although T cell precursors require specific cognate interaction for maturation, human $CD34^+CD4^+$ $CD8^+$ and $CD3^+CD2^+$ cells were generated in NOD-SCID mice BM following injection of $CD34^+CD38^-$ cells (Bhatia et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:5320-5325; Verstegen et al., 1998, Blood 91:1966-1976) or $CD34^-lin^-$ cells (Bhatia et al., 1998, Nature Med. 4:1038-1045). Also, *in vitro* experiments in the prior art indicate that the BM microenvironment is permissive for T cell development, and may recapitulate thymic maturation (Garcia-Ojeda et al., 1998, J. Exp. Med. 187:1813-1823). Further, without wishing to be bound by theory, the presence of contaminant mature human T cells in the transplanted $CD34^+KDR^+$ cells can be excluded in view of the lack of human T lymphocytes in mice receiving large numbers of $CD34^+KDR^-$ cells. Thus, the data disclosed herein demonstrate that human T cell precursors develop in BM of NOD-SCID mice. Taken together, these data establish that the $CD34^+KDR^+$ population, but not the $CD34^+KDR^-$ subset, is capable of establishing long-term (3 month) human hematopoiesis of the various hematopoietic lineages in NOD-SCID mice recipients.

NOD-SCID cord blood studies

In five independent experiments, 200 to 15,000 $CD34^+KDR^+$ or 10,000 to 200,000 $CD34^+KDR^-$ CB cells were xenotransplanted into NOD-SCID mice. Human cells were virtually absent from mice transplanted with double sorted KDR^- cells. In contrast, KDR^+ cells consistently generated human $CD45^+$ cells in BM, PB,

and spleen of the recipient mice according to a dose-dependent pattern, *e.g.*, representative results indicate that mice receiving 1,000 to 10,000 cells exhibited 27.2±7.1% (mean ± SEM) human $CD45^+$ BM cells, whereas animals receiving 200 to about 800 cells demonstrated 3.75±1.5% $CD45^+$ BM cells. In a representative experiment, mice transplanted with 6,000 $CD34^+KDR^+$ cells (Figure 3C) exhibited abundant BM human CD34 progenitors, precursors of the erythroid, granulomonocytic, and megakaryocytic lineages, as well as B and NK cells. The low CD3 expression detected may, without wishing to be bound by theory, reflect the low T cell generation potential of CB HSCs.

Multilineage engraftment of sheep fetuses using $CD34^+KDR^{+/+}$ cells

BM studies involving $CD34^+KDR^+$ cells similar to those performed in NOD-SCID mice and disclosed previously herein were also performed in fetal sheep.

In a representative experiment, $CD34^+$ cells were purified from two human BM samples. The $CD34^+KDR^{+/\pm}$ or the $CD34^+KDR^-$ subfraction was then injected into the pre-immune fetuses of eight pregnant ewes. The primary recipients received $CD34^+KDR^{+/\pm}$, $CD34^+KDR^-$, or $CD34^+$ cells (four, three and two fetuses per group, respectively) and the recipients were then sacrificed on day 60 post-transplant. Other fetuses injected with $CD34^+KDR^{+/\pm}$ or with $CD34^+KDR^-$ cells were born. In addition, human $CD34^+$ cells from primary fetuses treated with $KDR^{+/\pm}$ cells were transplanted into secondary fetuses (Kawashima et al., 1996, Blood 88:4136-4142; Civin et al., 1996, Blood 88:4102-4109).

In primary fetal sheep recipients, transplantation of 1.2×10^5 $CD34^+$ cells per fetus consistently induced engraftment; that is, BM analysis indicated the presence of a significant fraction of differentiated (0.30% $CD45^+$ cells, mean values) and undifferentiated (0.17% $CD34^+$ cells) hematopoietic precursors. Further, clonogenic assay demonstrated that 6.8% CFU-Mix/BFU-E and 5.2% CFU-GM of all scored colonies were of human origin. A small number (3×10^3 cells/fetus) of $CD34^+KDR^{+/\pm}$ cells consistently engrafted with an impressive multilineage

expression for the differentiated compartments: 1.78% $CD45^+$, 0.16% GPA^+, and 0.34% $CD3^+$ cells. Further, these fetuses exhibited a consistent engrafment with multilineage expression for the undifferentiated compartment: 0.32% $CD34^+$. Within the HPC pool, the frequency of human HPCs was elevated, *i.e.*, 9.3% for CFU-Mix/BFU-E and 16.2% for CFU-GM of all scored colonies were of human origin. An 80-fold larger number (2.4×10^5 cells/fetus) of $CD34^+KDR^-$ cells did not engraft any fetus, as indicated by the consistent absence of $CD34^+$ and $CD3^+$ cells. Moreover, only a small percentage of differentiated hematopoietic precursors was detected (*i.e.*, 0.7% CD45 cells), together with a few late CFU-GM (2.4%) giving rise to small colonies. It is estimated that an approximate total of greater than 10^8 $CD34^+$ and $CD3^+$ human cells were generated per fetus by KDR^+ cells, whereas no $CD34^+$ and $CD3^+$ cells were generated by KDR^- cells (Figure 4, middle and top panels).

Each secondary fetal sheep recipient received 4×10^5 human BM $CD34^+$ cells, derived from the primary fetuses originally transplanted with $KDR^{+/\pm}$ cells. After two months, the four secondary recipients were sacrificed and all demonstrated multi-lineage engraftment (Figure 4, bottom).

In born sheep recipients at three weeks after birth, both sheep transplanted with KDR cells in fetal life exhibited persistent multilineage engraftment at the BM level. One sheep featured an extremely abundant progeny of human $CD45^+$ cells and 8.8% colonies of human origin, and the other sheep exhibited 1.0% $CD45^+$ cells (the colony number was not evaluated for this sheep due to bacterial contamination of the culture plates).

These representative fetal sheep results, confirmed in other experiments, indicate that the $CD34^+KDR^{+/\pm}$ fraction is enriched for HSCs giving rise to multilineage engraftment in primary/secondary fetuses and born sheep. The engraftment in secondary recipients is noteworthy. Indeed, positive results in secondary fetal recipients successfully compare with those observed by follow up to primary transplanted fetuses for long periods after birth (Civin, 1996, Blood 88:4102-4109). On the other hand, the $CD34^+KDR^-$ fraction does not engraft and

contains only HPCs giving rise, in primary recipients, to differentiated hematopoietic precursors and a few late CFU-GM.

In sum, the data disclosed herein regarding the NOD-SCID and fetal sheep xenotransplantation assays indicate that restriction of HSCs to the KDR^+ subfraction of $CD34^+$ cells. Previous studies in NOD-SCID mice and in sheep fetuses demonstrated that HSCs are enriched in diverse $CD34^+$ cell subfractions, *e.g.*, $CD38^-$ (Bhatia et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:5320-5325; Wang et al., 1997, Blood 89:3919-3924; Conneally et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:9836-9841; Verstegen et al., 1998, Blood 91:1966-1976; Civin et al., 1996, Blood 88:4102-4109), kit^{low} (Kawashima et al., 1996, Blood 87:4136-4142), $Thy\text{-}1^+$ (Sutherland et al., 1996, Exp. Hematol. 24:795-806), and Rhodamine $(Rh)^{dim}$ (Uchida et al., 1996, Blood 88:1297-1305). However, engraftment was also observed at a lower level for the complementing subfractions, *i.e.*, $CD38^+$ cells (Conneally et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:9836-9841; Verstegen et al., 1998, Blood 91:1966-1976; Civin et al., 1996, Blood 88:4102-4109), kit^- (Kawashima et al., 1996, Blood 87:4136-4142), $Thy\text{-}1^-$ (Sutherland et al., 1996, Exp. Hematol. 24:795-806), and Rh^{bright} (Uchida et al., 1996, Blood 88:1297-1305).

Frequency of repopulating HSCs and 12-week CAFCs/LTC-ICs in $CD34^+KDR^+$ cell fraction

In NOD-SCID mice injected with from about 100 to about 1,600 BM $CD34^+KDR^+$ cells, the representative $CD45^+$ cell dose-response (Figure 3A) indicated that a cell number far lower than 100 cells would successfully engraft. Therefore, a representative LDA was performed using 250, 50, 10 or 5 BM $CD34^+KDR^+$ cells/mouse (Figure 3B). After injection of 250 to 5 BM KDR^+ cells, a dose-dependent multilineage engraftment was detected (Figure 3B, top and bottom left). All mice were repopulated by 250 and 50 cells, while five of six mice injected with 10 cells and four of 6 mice injected with 5 cells were engrafted based on flow cytometry analysis (Figure 3B, top left) and HPC assay validated by PCR of human a-satellite DNA in the scored colonies (Figure 3B, bottom). LDA indicated an

approximately 20% frequency value for repopulating HSCs in $CD34^+KDR^+$ cells (Figure 3B, top right). This representative value is similar to the representative 25% CAFC frequency exhibited in $VEGF^-$ BM LTC, indicating that repopulating HSCs and 12 week LTC-ICs/CAFCs are closely related.

In representative experiments on 12 week extended LTCs treated or not with VEGF, LDA indicated that the CAFC frequency in $CD34^+KDR^+$ cell of BM (Figure 2B, left) or CB (Figure 2B, right) CAFC is lower in $VEGF^-$ (approximately 25-35%) than in $VEGF^+$ (approximately 53-61%) LTC. No CAFC were detected in $CD34^+KDR^-$ cell fractions.

Representative corresponding experiments on LTC-IC frequency in $CD34^+KDR^-$ or $CD34^+KDR^+$ fractions from BM, CB, MPB and PB showed a pattern similar to that observed for CAFC frequency.

The 20% repopulating HSCs frequency in $CD34^+KDR^+$ BM cells was about 100-150-fold more elevated than the frequency reported in $CD34^+CD38^-$ BM or CB cells (Bhatia et al., 1997, Proc. Natl. Acad. Scid. U.S.A. 94:5320-5325; Wang et al., 1997, Blood 89:3919-3924; Conneally et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:9836-9841). It is noteworthy that in representative experiments the $CD34^+CD38^-$ fraction comprises about 1-2% KDR^+ cells. This result explains the different HSC frequency in the $CD34^+38^-$ subset compared to the frequency in the $CD34^+KDR^+$ cell subset. The assay performed herein lasted for 3 months and the mice were not treated with cytokines, whereas in other studies the assay usually lasts 1.5 to 2 months and often involves cytokine treatment (Larochelle et al., 1996, Nature Med. 2:1329-1337).

Representative *in vitro* LDAs indicated that 25 to 35% CAFCs were present in BM and CB $CD34^+KDR^+$ cells, as evaluated in $VEGF^-$ 12-week LTC. Without wishing to be bound by theory, since the CAFC frequency rises to 53 to 63% in these representative VEGF+ LTCs, it is predicted that the *in vivo* repopulating HSC frequency will be more elevated in mice injected with human VEGF ± with or without other cytokines. Importantly, the significant increase of CAFC/LTC-IC frequency

induced by VEGF addition suggests that VEGF exerts a key proliferative and/or anti-apoptotic effect on putative HSCs.

Increased 12 week CAFC/LTC-IC frequency in starvation resistant $CD34^+KDR^+$ cells

The 12 week LTC-IC frequency in starvation resistant $CD34^+KDR^+$ or $CD34^+KDR^{+/\pm}$ cells was examined. In representative experiments, $CD34^+KDR^+$ or $CD34^+KDR^{+/\pm}$ and $CD34^+KDR^-$ cells were seeded into FCS^- free liquid suspension minibulk cultures, supplemented with VEGF but deprived of other HGFs. The KDR^+ or $KDR^{+/\pm}$ cell number decreased sharply in the first five days of culture, but then leveled down to 10-25% residual cells through day 30. Conversely, all KDR^- cells were dead at day 10 of culture. In single $CD34^+KDR^+$ cell starvation cultures not supplemented by VEGF all cells died while approximately 20% of cells treated with VEGF survived (Figure 2D, top), indicating the key anti-apoptotic effect of VEGF on this cell type.

The starvation resistant $KDR^{+/\pm}$ fraction contained virtually no multipotent/primitive HPCs (CFU-Mix/HPP-CFC assays), but exhibited an elevated 12 week LTC-IC frequency, approximately ≥80-95% at day 5-30 (Figure 2D, bottom panels). Control KDR^- cells never contained 12 week LTC-ICs. Without wishing to be bound by theory, based on the similarity between *in vivo* and *in vitro* HSC assay results, it may be that the starvation-resistant $CD34^+KDR^+$ cells represent HSCs having *in vivo* long-term repopulating capacity. The data disclosed herein are in accord with prior studies demonstrating that one of the key features of adult HSCs is their quiescent status in a prolonged cell cycle (Ogawa, 1993, Blood 81:2844-2853; Morrison et al., 1997, Cell 88:287-298; Orlic and Bodine, 1994, Blood 84:3991-3994). That is, the high frequency of HSCs in $CD34^+KDR^+$ cells capable of withstanding serum starvation may be due to their ability to remain quiescent which is a known characteristic of adult HSCs thus further suggesting that KDR^+ is a marker specific for HSCs.

HSCs in $CD34^-/lin^-/KDR^+$ cells

Experimental and clinical observations leave little doubt that human HSCs with long-term engrafting ability are $CD34^+$ (Berenson et al., J. Clin. Invest. 81:951-955; Berenson et al., 1991, Blood 77:1717-1722; Bensinger et al., 1996, Blood 88:4132-4138). This has also been confirmed not only in the SCID mouse models, but also in the sheep models where $CD34^+$ cells have caused engraftment lasting >5 years (Zanjani et al., 1996, Int. J. Hematol. 63:179-192). However, recent studies in both mice (Osawa et al., 1996, Science 273:242-245; Goodell et al., 1996, J. Exp. Med. 183:1797-1806) and rhesus monkeys (Johnson et al., 1996, Blood 88:629a) have demonstrated the $CD34^-$ cells population contain progenitors capable of producing $CD34^+$ cells *in vitro* and to be highly enriched in HSCs with competitive long-term *in vivo* repopulating potential.

Recent reports (Zanjani et al., 1998, Exp. Hematol. 26:353-360; Almeida-Porada et al., 1998, Exp. Hematol. 26:749) suggest that in the sheep fetus large numbers ($>10^5$) of human BM $CD34^-$ cells can engraft.

Furthermore, studies by Bhatia et al. (1998, Nature Med.4:1038-1045) indicate that 1-2 x 10^5 BM or CB $CD34^-lin^-$ cells engraft a majority of NOD-SCID mice after 2-3 months, with generation of $CD34^+$ cells and multilineage expression including B and T lymphocytes. The data disclosed herein demonstrate that NOD-SCID mice injected with 4,000 $CD34^-lin^-$ KDR^+ CB cells consistently exhibited $CD34^+$ cell generation and multilineage engraftment after three months. Specifically, the following representative values were detected in BM: 0.19% $CD34^+$ and 0.11% $CD34^+CD45^+$ cells, coupled with multilineage expression (*e.g.*, 0.23% $CD45^+$, 0.18% $CD33^+$, 0.10% $CD15^+$, 0.27% GPA^+, 0.27% $CD71^+$, 0.15% $CD20^+$, 0.12% $CD19^+$, 0.25% $CD3^+$, and 0.11% $CD56+CD16^+$). In the same experiment, 4,000 $CD34^-KDR^+$ cells engrafted. Furthermore, 10,000 KDR^+ CB mononuclear cells engrafted, whereas 100,000 KDR^- CB mononuclear cells did not engraft.

A large number of human BM and CB $CD34^-lin^-$ cells engraft fetal sheep and NOD-SCID mice, as indicated by multilineage expression and generation of a $CD34^+$ cells. Approximately one percent or less of $CD34^-/lin^-$ cells are KDR^+.

Indeed, a discrete number of CB $CD34^-lin^-$ KDR^+ cells engraft NOD-SCID mice and generate $CD34^+$ cells. Based on these results, and without wishing to be bound by theory, KDR is a key marker for $CD34^-$ HSC in post-natal life.

Although HSCs have previously been enriched in diverse $CD34^+$ cell subsets, a HSC defining marker had not, prior to the present invention, been identified. The data disclosed herein demonstrate that the $CD34^+KDR^+$ cell fraction has novel properties. HSCs are essentially restricted to this population, whereas oligo-unipotent HPCs are virtually restricted to $CD34^+KDR^-$ cells. Further, the HSC enrichment in $CD34^+KDR^+$ cells is strikingly elevated, *i.e.*, the putative HSC frequency rises to ≥80-95% in starvation resistant $CD34^+KDR^+$ cells. Altogether, these results indicate that KDR is a novel functional marker defining HSCs.

Purification of $CD34^+$ HPCs has markedly facilitated studies on early hematopoietic precursors (Ogawa et al., 1993, Blood 81:2844-2853; Gabbianelli et al., 1990, Science 249:1561-1564). The isolation of KDR^+ HSCs offers a unique opportunity to elucidate the cellular/molecular phenotype and functional properties of HSCs/HSC subsets. These issues, exceedingly elusive so far, are of pivotal significance for a large array of biotechnological and clinical aspects, *e.g.*, autologous/allogeneic HSC transplantation, *in vitro* blood cell generation for transfusion medicine, and HSC gene therapy in hereditary/acquired hematology-immunology disorders.

The data disclosed herein shed light on recent studies on embryonic hematoangiogenesis. Studies on $Flk\text{-}1^{-/-}$ knock out mice (Shalaby et al., 1997, Cell 89:981-990) indicate that Flk-1 is required to initiate both primitive and definitive hematolymphopoiesis, as well as vasculogenesis. These data suggest a role for Flk-1 in generation of hemoangioblasts, *i.e.*, putative stem cells for both hematolymphopoietic and endothelial lineages (Flamme et al., 1992, Development 116:435-439). $Flk\text{-}1^+$ and $CD34^+$ cells are present in murine embryonic-fetal liver (Kabrun et al., 1997, Development 124:2039-2048). In differentiating embryonic stem cells, embryoid bodies treated with VEGF and KL give rise to $CD34^+$ and $flk\text{-}1^+$ blast cell colonies,

which generate secondary colonies composed of all hematopoietic lineages (Kennedy et al., 1997, Nature 386:488-492) and which also exhibit endothelial developmental capacity (Nishikawa et al., 1998, Development 125:1747-1757; Choi et al., 1998, Development 125:725-732).

Altogether, previous studies suggested the existence of embryonic $CD34^+flk\text{-}1^+$ hemoangioblast, but did not provide evidence for a prenatal $CD34^+flk\text{-}1^+$ repopulating HSC. The data disclosed herein demonstrate the existence of post-natal $CD34^+KDR^+$ repopulating HSC. Without wishing to be bound by theory, taking together the data disclosed herein, KDR-flk-1 may hypothetically define both post-natal and pre-natal HSCs/hemoangioblasts.

Recently, bone marrow-derived cells have been demonstrated to give rise to hepatic oval cells, which can differentiate into the other two types of epithelial cells in the liver, *i.e.*, ductular cells and hepatocytes (Petersen et al., 1999, Science 284:1168-1170). In addition, bone marrow-derived cells have been demonstrated to have the capability to give rise to myogenic progenitors (Ferrari et al., 1998, Science 279:1528-1530). Also, bone marrow-derived were induced to differentiate into the adipocytic, chondrocytic, or osteocytic lineages (Pittenger et al., 1999, Science 284:143-147). Without wishing to be bound by theory, it appears that the stem cells giving rise to epithelial liver cell progenitors, myogenic progenitors, and/or bone, cartilage, fat, tendon, and marrow stromal cells is the KDR^+ stem cell population of the present invention. Thus, the present invention provides methods of isolating and purifying cells which not only give rise to multilineage hematopoietic engraftment, but may also provide methods of targeting gene therapies to a wide variety of tissues including muscle and liver. Therefore, the prior art has only tantalized in suggesting that such multipotent cells existed, however, only the present invention teaches how to obtain them.

In summary, the major hurdle in studies on hematolymphopoietic stem cells (HSCs) has been the lack of an HSC-specific marker. The lack of a specific HSC marker hampered the purification, characterization and utilization of this extremely rare

cell population. The data disclosed herein demonstrate, for the first time, that the vascular endothelial growth factor receptor 2 (VEGFR2, KDR/Flk-1) is a specific functional marker for human HSCs in adult bone marrow (BM), normal or mobilized peripheral blood (PB, MPB), and cord blood (CB). In these post-natal tissues, pluripotent repopulating HSCs are virtually restricted to and highly purified in the miniscule $CD34^+KDR^+$ cell fraction (<1% of $CD34^+$ cells), as evaluated in NOD-SCID mice and fetal sheep xenografts. This $CD34^+KDR^+$ cell fraction contains essentially no oligo-unipotent hematopoietic progenitor cells (HPCs). Conversely, oligo-unipotent HPCs are virtually restricted to and highly purified in $CD34^+KDR^-$ cells, which contain essentially no HSCs.

In a representative experiment, the frequency of repopulating HSCs in the BM $CD34^+KDR^+$ subset, evaluated in NOD-SCID mice by limiting dilution assay (LDA), is 20%; similarly, representative experiments showed that the frequency of putative HSCs (CAFC) in the BM $CD34^+KDR^+$ subset, evaluated by LDA in 12-week extended Dexter-type long term culture (LTC), was 25%. The frequency rose in LTC supplemented with VEGF (to 53% in representative experiments), thus suggesting a functional role for the VEGF/KDR system in HSCs. Conversely, putative HSCs were essentially not detected in the $CD34^+KDR^-$ subset. In addition, the fraction of $CD34^+KDR^+$ cells resistant to prolonged GF starvation (except for VEGF addition) in FCS^- free culture comprises a very elevated frequency of putative HSCs, ≥ 80-95% in representative experiments.

The data disclosed herein indicate that KDR is a functional HSC defining marker, which distinguishes HSCs from oligo-unipotent HPCs. The present invention makes possible the characterization and functional manipulation of HSCs/HSC subsets, as well development of innovative approaches for HSC clinical utilization.

The disclosures of each and every patent, application and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A method of obtaining a cell population enriched for long-term repopulating human hematopoietic stem cells, said method comprising obtaining a population of cells from human hematopoietic tissue and isolating a population of KDR^+ cells therefrom, thereby obtaining a cell population enriched for long-term repopulating human hematopoietic stem cells.

2. The method of claim 1, wherein said human hematopoietic tissue is selected from the group consisting of pre-embryonic hematopoietic tissue, embryonic hematopoietic tissue, fetal hematopoietic tissue, and post-natal hematopoietic tissue.

3. The method of claim 2, wherein said embryonic hematopoietic tissue is selected from the group consisting of yolk sac, and embryonic liver.

4. The method of claim 2, wherein said fetal hematopoietic tissue is selected from the group consisting of fetal liver, fetal bone marrow and fetal peripheral blood.

5. The method of claim 2, wherein said post-natal hematopoietic tissue is selected from the group consisting of cord blood, bone marrow, normal peripheral blood, mobilized peripheral blood, hepatic hematopoietic tissue, and splenic hematopoietic tissue.

6. The method of claim 1, wherein said KDR^+ cells are isolated using a reagent which specifically binds KDR.

7. The method of claim 6, wherein said reagent is an antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

8. The method of claim 7, wherein said antibody is a monoclonal antibody.

9. The method of claim 8, wherein said monoclonal antibody is 260.4.

10. The method of claim 1, wherein said KDR^+ cells are isolated using a conjugated vascular epithelial growth factor or a molecule derived therefrom.

11. The method of claim 1, wherein said cells are starvation resistant long-term repopulating human hematopoietic stem cells.

12. An enriched population of long-term repopulating human hematopoietic stem cells obtained using the method of claim 1.

13. A cell obtained using the method of claim 1.

14. The cell of claim 13, wherein said cell comprises an isolated nucleic acid.

15. The cell of claim 14, wherein said isolated nucleic acid is selected from the group consisting of a nucleic acid encoding adenosine deamininase, a nucleic acid encoding β-globin, a nucleic acid encoding multiple drug resistance, an antisense nucleic acid complementary to a human immunodeficiency virus nucleic acid, an antisense nucleic acid complementary to a nucleic acid encoding a cell cycle gene, and an antisense nucleic acid complementary to a nucleic acid encoding an oncogene.

16. The cell of claim 14, wherein said isolated nucleic acid is operably linked to a promoter/regulatory sequence.

17. The cell of claim 16, wherein said promoter/regulatory sequence is selected from the group consisting of a retroviral long terminal repeat, and the cytomegalovirus immediate early promoter.

18. A method of obtaining a purified population of long-term repopulating human hematopoietic stem cells, said method comprising obtaining a population of cells from human hematopoietic tissue, isolating a population of hematopoietic progenitor cells therefrom, and isolating a population of KDR^+ cells from said population of hematopoietic progenitor cells, thereby obtaining a purified population of long-term repopulating human hematopoietic stem cells.

19. The method of claim 18, wherein said human hematopoietic tissue is selected from the group consisting of pre-embryonic hematopoietic tissue, embryonic hematopoietic tissue, fetal hematopoietic tissue, and post-natal hematopoietic tissue.

20. The method of claim 19, wherein said embryonic hematopoietic tissue is selected from the group consisting of yolk sac, and embryonic liver.

21. The method of claim 19, wherein said fetal hematopoietic tissue is selected from the group consisting of fetal liver, fetal bone marrow and fetal peripheral blood.

22. The method of claim 19, wherein said post-natal hematopoietic tissue is selected from the group consisting of cord blood, bone marrow, normal peripheral blood, mobilized peripheral blood, hepatic hematopoietic tissue, and splenic hematopoietic tissue.

23. The method of claim 18, wherein said hematopoietic progenitor cells are isolated using at least one method selected from the group consisting of isolation of cells expressing an early marker using antibodies specific for said marker, isolation of cells not expressing a late marker using antibodies specific for said late marker, isolation of cells based on a physical property of said cells, and isolation of cells based on a biochemical/biological property of said cells.

24. The method of claim 23, wherein said early marker is selected from the group consisting of CD34, Thy-1, c-kit receptor, flt3 receptor, AC133, vascular endothelial growth factor receptor I, vascular endothelial growth factor receptor III, Tie1, Tek, and basic fibroblast growth factor receptor.

25. The method of claim 23, wherein said late marker is a lineage (lin) marker.

26. The method of claim 24, wherein said early marker is CD34.

27. The method of claim 26, wherein said hematopoietic progenitor cells are obtained from said hematopoietic tissue using an antibody which specifically binds CD34 to select a population of $CD34^+$ hematopoietic progenitor cells.

28. The method of claim 27, wherein said population of KDR^+ cells is isolated from said population of $CD34^+$ hematopoietic progenitor cells using an antibody which specifically binds KDR.

29. The method of claim 28, wherein said antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

30. The method of claim 29, wherein said antibody is a monoclonal antibody.

31. The method of claim 30, wherein said monoclonal antibody is 260.4.

32. The method of claim 31, wherein said cells are starvation resistant human hematopoietic stem cells.

33. An isolated purified population of long-term repopulating human hematopoietic stem cells obtained by the method of claim 17.

34. A cell obtained by the method of claim 17.

35. The cell of claim 34, wherein said cell comprises an isolated nucleic acid.

36. The cell of claim 35, wherein said isolated nucleic acid is selected from the group consisting of a nucleic acid encoding adenosine deaminase, a nucleic acid encoding β-globin, a nucleic acid encoding multiple drug resistance, an antisense nucleic acid complementary to a human immunodeficiency virus nucleic acid, an antisense nucleic acid complementary to a nucleic acid encoding a cell cycle gene, and an antisense nucleic acid complementary to a nucleic acid encoding an oncogene.

37. The cell of claim 35, wherein said isolated nucleic acid is operably linked to a promoter/regulatory sequence.

38. The cell of claim 37, wherein said promoter/regulatory sequence is selected from the group consisting of a retroviral long terminal repeat, and the cytomegalovirus immediate early promoter.

39. The method of claim 26, wherein said hematopoietic progenitor cells are obtained from said hematopoietic tissue using antibody which specifically binds CD34 to select a population of $CD34^-$ cells.

40. The method of claim 39, wherein said hematopoietic progenitor cells are obtained from said population of $CD34^-$ cells using antibody which specifically binds lin to select a population of $CD34^-lin^-$ cells.

41. The method of claim 40, wherein said population of KDR^+ cells is isolated from said population of $CD34^-lin^-$ cells using an antibody which specifically binds KDR.

42. The method of claim 41, wherein said antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

43. The method of claim 42, wherein said antibody is a monoclonal antibody.

44. The method of claim 43, wherein said monoclonal antibody is 260.4.

45. A purified population of long-term repopulating human hematopoietic stem cells obtained by the method of claim 41.

46. A cell isolated by the method of claim 41.

47. The cell of claim 46, wherein said cell comprises an isolated nucleic acid.

48. The cell of claim 47, wherein said isolated nucleic acid is selected from the group consisting of a nucleic acid encoding adenosine deaminase, a nucleic acid encoding β-globin, a nucleic acid encoding multiple drug resistance, an antisense nucleic acid complementary to a human immunodeficiency virus nucleic acid, an antisense nucleic acid complementary to a nucleic acid encoding a cell cycle gene, and an antisense nucleic acid complementary to a nucleic acid encoding an oncogene.

49. The cell of claim 48, wherein said isolated nucleic acid is operably linked to a promoter/regulatory sequence.

50. The cell of claim 49, wherein said promoter/regulatory sequence is selected from the group consisting of a retroviral long terminal repeat, and the cytomegalovirus immediate early promoter.

51. A method of expanding a population of long-term repopulating human hematopoietic stem cells, the method comprising obtaining a population of cells from human hematopoietic tissue, isolating a population of KDR^+ hematopoietic stem cells therefrom, and incubating said population of KDR^+ cells with vascular endothelial growth factor, thereby expanding said population of long-term repopulating human hematopoietic stem cells.

52. The method of claim 51, further comprising incubating said population of KDR^+ cells with at least one growth factor.

53. The method of claim 52, wherein said growth factor is selected from the group consisting of flt3 receptor ligand, kit receptor ligand, thrombopoietin,

basic fibroblast growth factor, interleukin 6, interleukin 11, interleukin 3, granulomonocytic colony-stimulatory factor, granulocytic colony-stimulatory factor, monocytic colony-stimulatory factor, erythropoietin, angiopoietin, and hepatocyte growth factor.

54. An isolated purified population of long-term repopulating human hematopoietic stem cells obtained by the method of claim 51.

55. A cell obtained using the method of claim 51.

56. The cell of claim 55, wherein said cell comprises an isolated nucleic acid.

57. The cell of claim 56, wherein said isolated nucleic acid is selected from the group consisting of a nucleic acid encoding adenosine deaminase, a nucleic acid encoding β-globin, a nucleic acid encoding multiple drug resistance, an antisense nucleic acid complementary to a human immunodeficiency virus nucleic acid, an antisense nucleic acid complementary to a nucleic acid encoding a cell cycle gene, and an antisense nucleic acid complementary to a nucleic acid encoding an oncogene.

58. The cell of claim 57, wherein said isolated nucleic acid is operably linked to a promoter/regulatory sequence.

59. The cell of claim 58, wherein said promoter/regulatory sequence is selected from the group consisting of a retroviral long terminal repeat, and the cytomegalovirus immediate early promoter.

60. A blood substitute comprising the progeny cells of an isolated purified population of long term repopulating human hematopoietic stem cells.

61. The blood substitute of claim 60, wherein said progeny cells are selected from the group consisting of red blood cells, neutrophilic granulocytes, eosinophilic granulocytes, basophilic granulocytes, monocytes, dendritic cells, platelets, B lymphocytes, T lymphocytes, natural killer cells, and differentiated precursors thereof, and undifferentiated progenitors thereof.

62. A chimeric non-human mammal comprising at least one of an isolated and purified long-term repopulating human hematopoietic stem cell.

63. The chimeric mammal of claim 62, wherein said cell is introduced into said mammal using a method selected from the group consisting of transplantation, and blastocyst injection.

64. The non-human mammal of claim 63, wherein said mammal is selected from the group consisting of a mouse, a rat, a dog, a donkey, a sheep, a pig, a horse, a cow, a non-human primate.

65. A method of inhibiting rejection of a transplanted organ, said method comprising ablating the bone marrow of a transplant recipient and administering to said recipient a multi-lineage engrafting dose of an isolated and purified long-term repopulating human hematopoietic stem cell obtained from the hematopoietic tissue of the donor of said organ, thereby inhibiting rejection of a transplanted organ.

66. A method of transplanting an autologous human hematopoietic stem cell in a human, said method comprising obtaining a population of cells from the hematopoietic tissue of a human and isolating a population of non-malignant hematopoietic stem cells therefrom, ablating the bone marrow of said human, and administering at least one said isolated non-malignant hematopoietic stem cell to said

human, thereby transplanting an autologous human hematopoietic stem cell in a human.

67. A method of isolating a KDR^+ cell, said method comprising selecting a cell expressing an antigen coexpressed with KDR, thereby isolating a KDR+ cell.

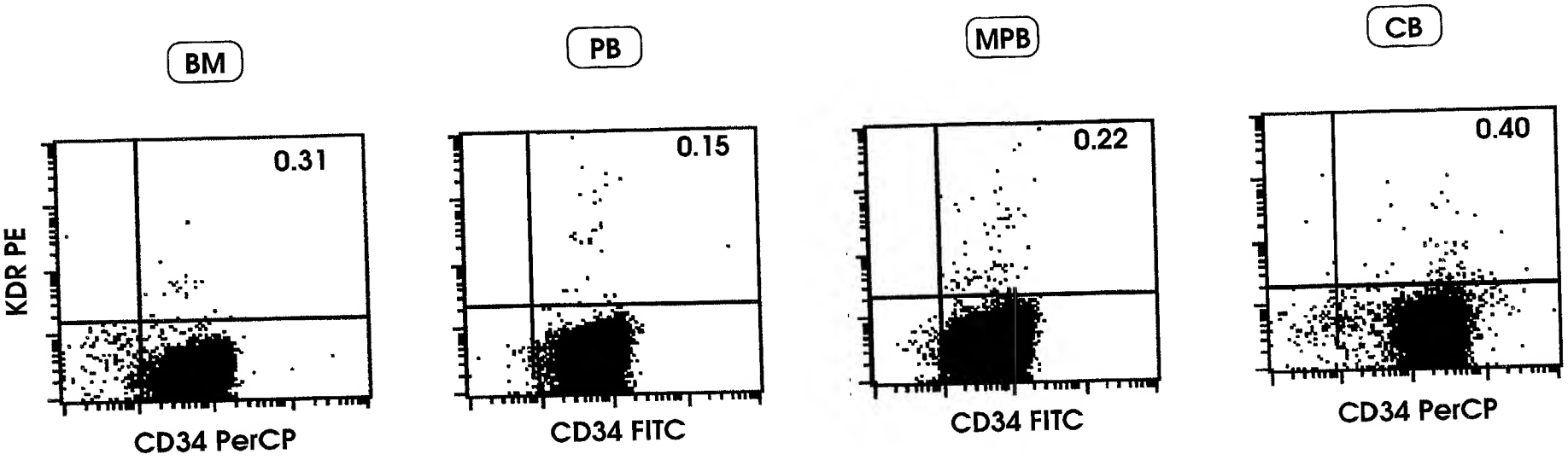
68. The method of claim 67, wherein said coexpressed antigen is selected from the group consisting of a vascular endothelial growth factor receptor I, and a vascular endothelial growth factor receptor III.

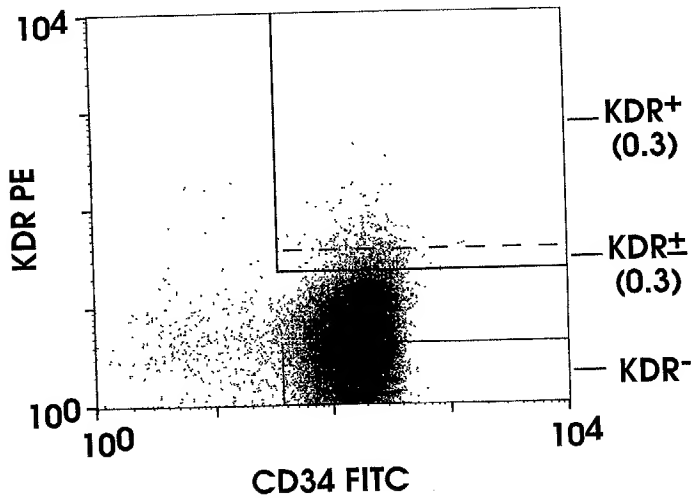
69. A method of isolating a KDR+ stem cell giving rise to at least one of a muscle cell, a hepatic oval cell, a bone cell, a cartilage cell, a fat cell, a tendon cell, and a marrow stroma cell said method comprising isolating a KDR+ stem cell from hematopoietic tissue, thereby isolating a KDR+ stem cell giving rise to at least one of a muscle cell, a hepatic oval cell, a bone cell, a cartilage cell, a fat cell, a tendon cell, and a marrow stroma cell.

70. A method of monitoring the presence of KDR+ stem cells in a human hematopoietic tissue in a human receiving therapy, said method comprising obtaining a sample of hematopoietic tissue from said human before, during and after said therapy, and measuring the number of KDR+ stem cells in said sample, thereby monitoring the presence of KDR+ stem cells in a human hematopoietic tissue obtained from a human receiving therapy.

ABSTRACT OF THE DISCLOSURE

The invention relates to a methods of obtaining and expanding a purified population of long-term repopulating hematopoietic stem cells. The invention also relates to the uses of a purified population of long-term repopulating hematopoietic stem cells.


BM
PB
MPB
CB
KDR PE
0.31
0.15
0.22
0.40
CD34 PerCP
CD34 FITC
CD34 FITC
CD34 PerCP


10⁴
10⁰
10⁰
10⁴
KDR PE
CD34 FITC
KDR+ (0.3)
KDR± (0.3)
KDR-

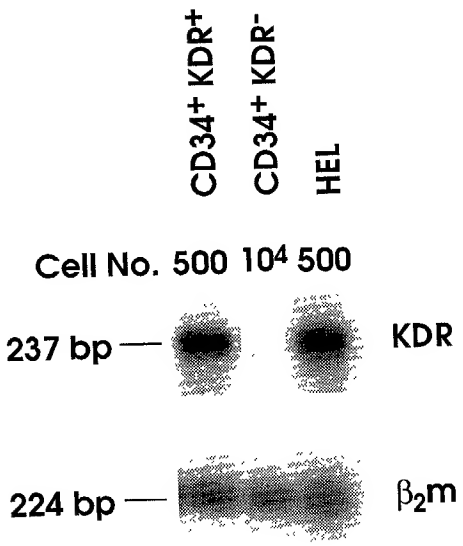

CD34+ KDR+
CD34+ KDR-
HEL
Cell No. 500 10⁴ 500
237 bp
KDR
224 bp
β2m

Fig. 1A

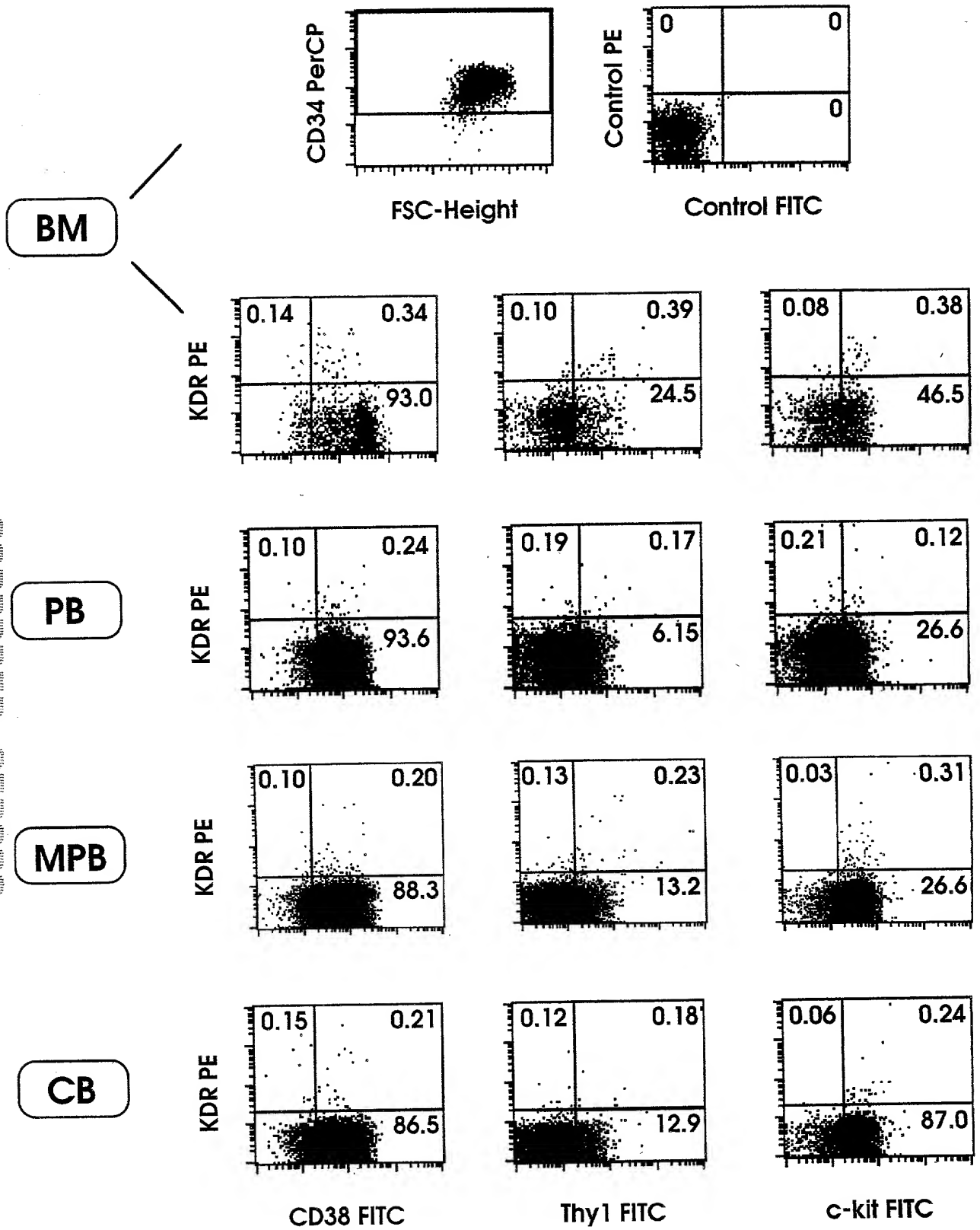

CD34 PerCP
FSC-Height
Control PE
Control FITC
0
0
0
0
BM
KDR PE
0.14
0.34
93.0
0.10
0.39
24.5
0.08
0.38
46.5
PB
KDR PE
0.10
0.24
93.6
0.19
0.17
6.15
0.21
0.12
26.6
MPB
KDR PE
0.10
0.20
88.3
0.13
0.23
13.2
0.03
0.31
26.6
CB
KDR PE
0.15
0.21
86.5
0.12
0.18
12.9
0.06
0.24
87.0
CD38 FITC
Thy1 FITC
c-kit FITC

Fig. 1B

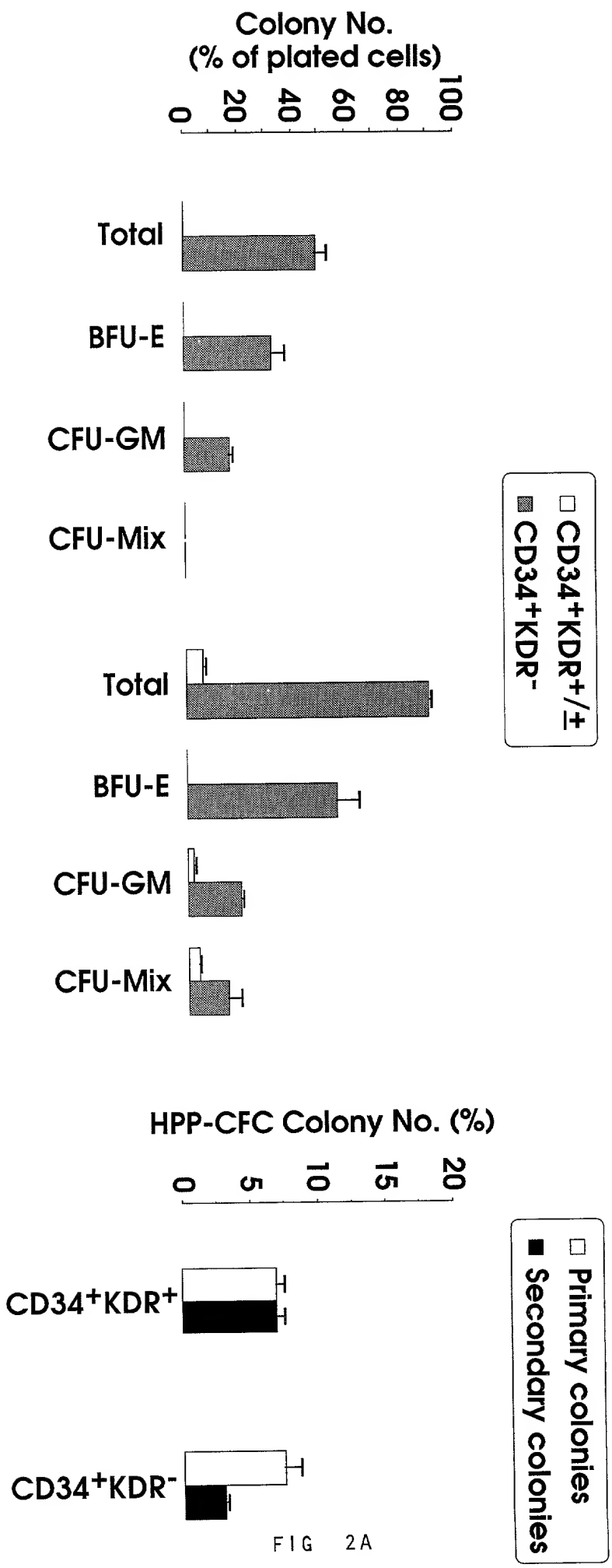

Colony No.
(% of plated cells)
0
20
40
60
80
100
Total
BFU-E
CFU-GM
CFU-Mix
Total
BFU-E
CFU-GM
CFU-Mix
□ CD34+KDR+/±
■ CD34+KDR-
HPP-CFC Colony No. (%)
0
5
10
15
20
CD34+KDR+
CD34+KDR-
□ Primary colonies
■ Secondary colonies

FIG 2A

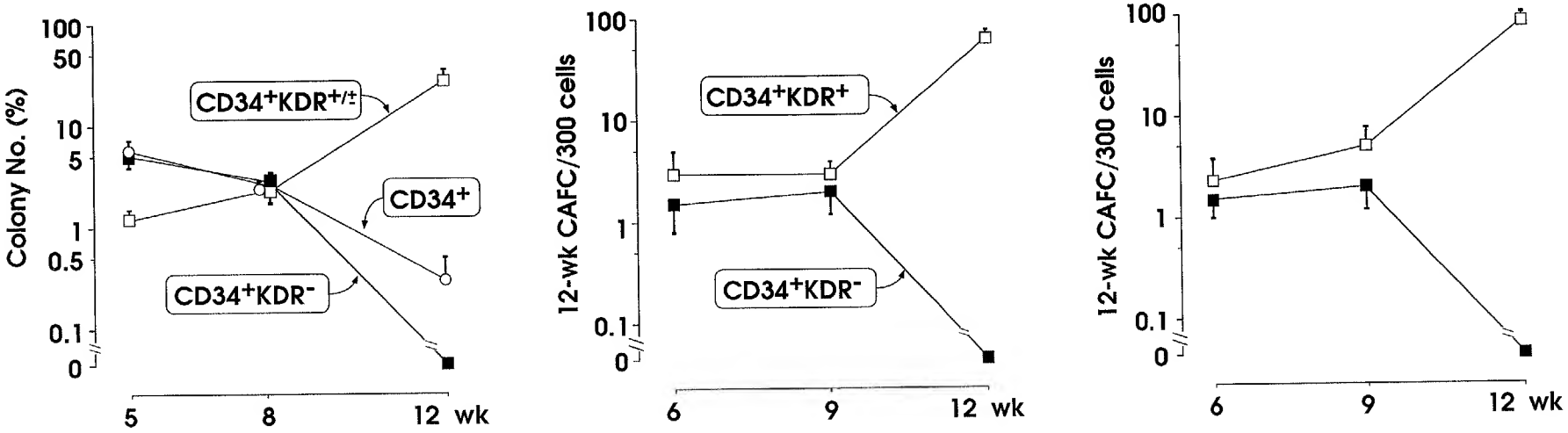
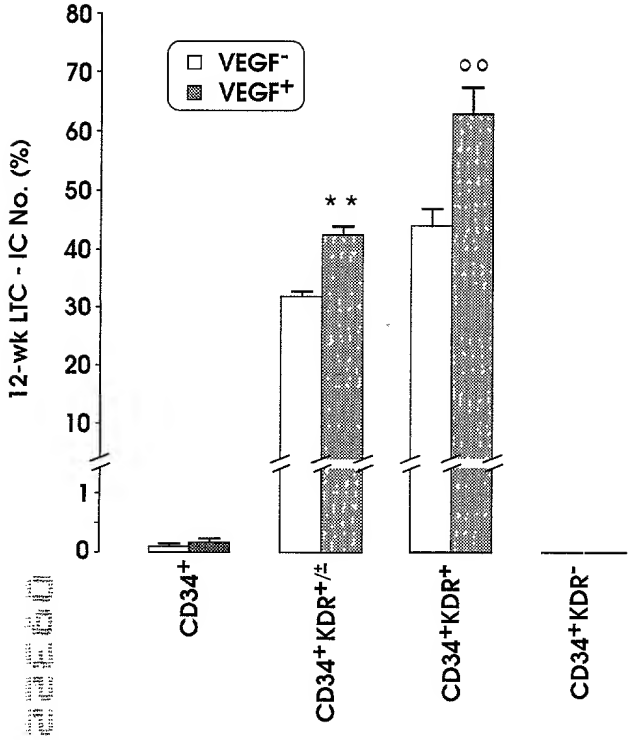
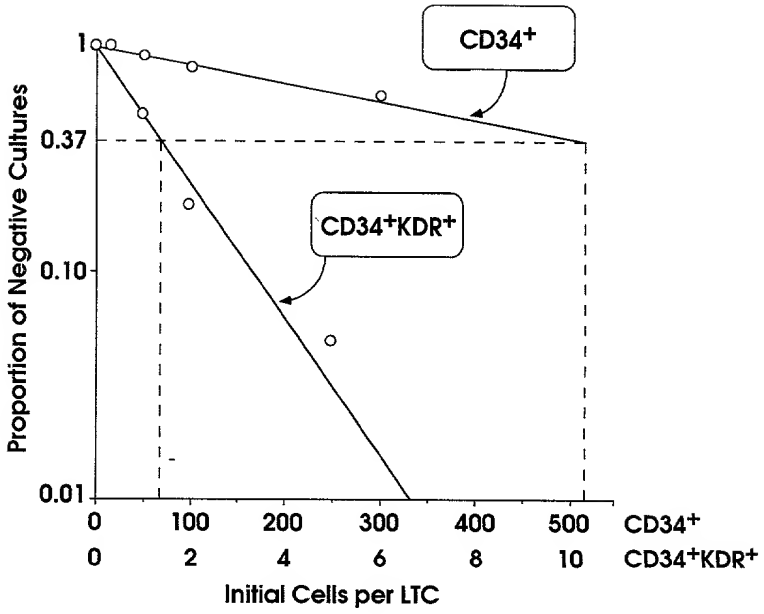
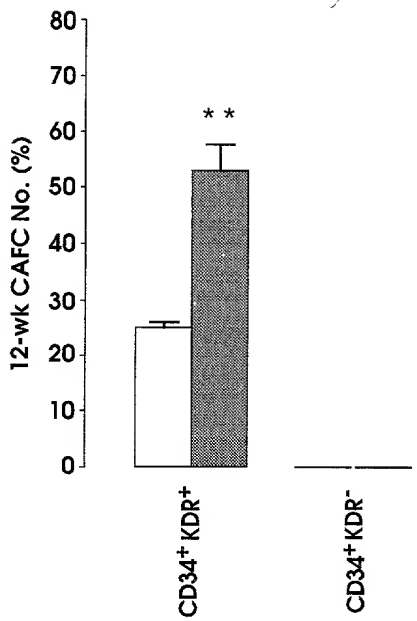

Colony No. (%)
100
50
10
5
1
0.5
0.1
0
CD34+KDR+/±
CD34+
CD34+KDR-
5
8
12 wk
12-wk CAFC/300 cells
100
10
1
0.1
0
CD34+KDR+
CD34+KDR-
6
9
12 wk
12-wk CAFC/300 cells
100
10
1
0.1
0
6
9
12 wk

Fig. 2B


12-wk LTC - IC No. (%)
VEGF-
VEGF+
80
70
60
50
40
30
20
10
1
0
* *
°°
CD34+
CD34+ KDR+/±
CD34+KDR+
CD34+KDR-


Proportion of Negative Cultures
1
0.37
0.10
0.01
CD34+
CD34+KDR+
0
100
200
300
400
500
CD34+
0
2
4
6
8
10
CD34+KDR+
Initial Cells per LTC


12-wk CAFC No. (%)
80
70
60
50
40
30
20
10
0
* *
CD34+ KDR+
CD34+ KDR-

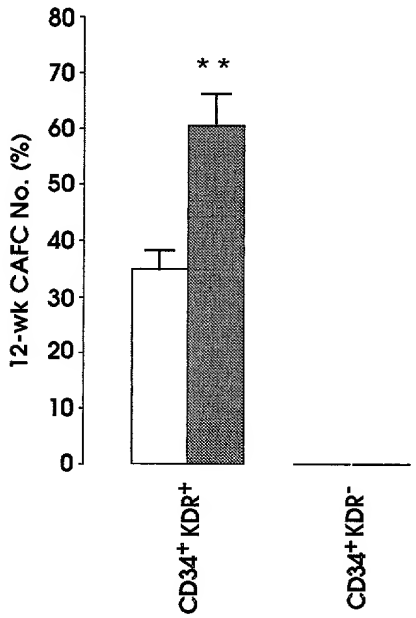

12-wk CAFC No. (%)
80
70
60
50
40
30
20
10
0
* *
CD34+ KDR+
CD34+ KDR-

Fig. 2C

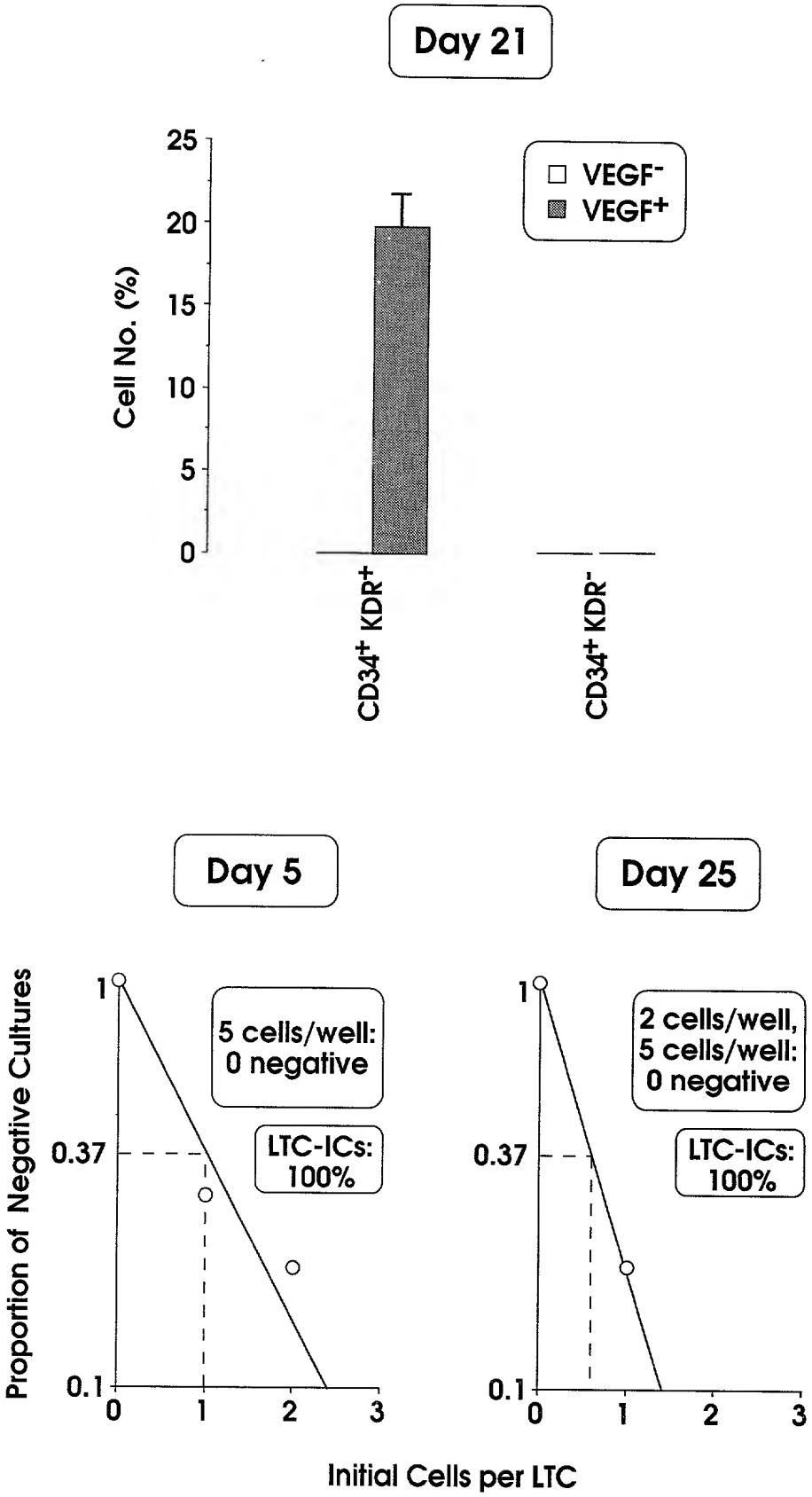

Day 21
25
20
15
10
5
0
Cell No. (%)
VEGF-
VEGF+
CD34+ KDR+
CD34+ KDR-
Day 5
Day 25
Proportion of Negative Cultures
1
0.37
0.1
0
1
2
3
5 cells/well:
0 negative
LTC-ICs:
100%
2 cells/well,
5 cells/well:
0 negative
LTC-ICs:
100%
Initial Cells per LTC

Fig. 2D

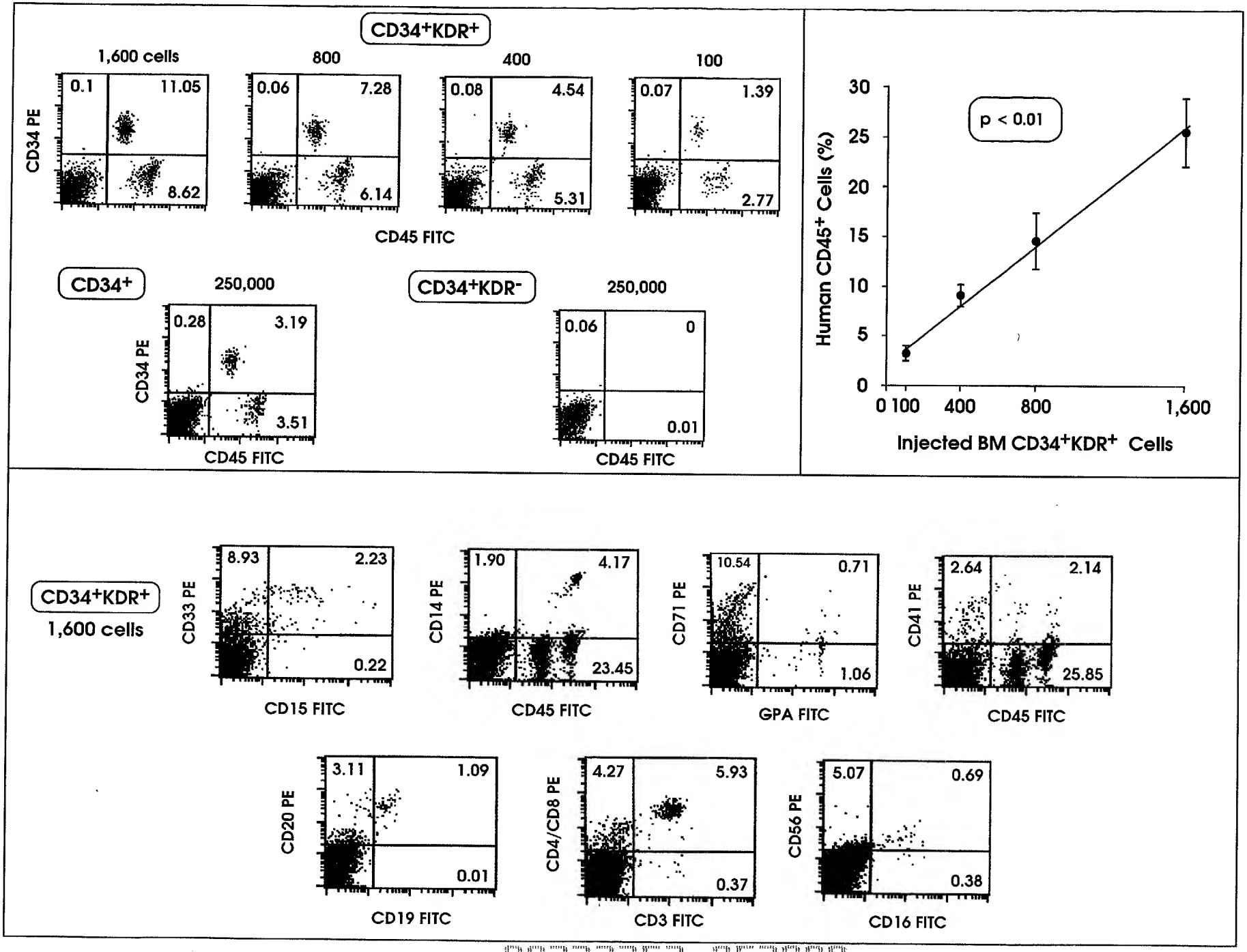
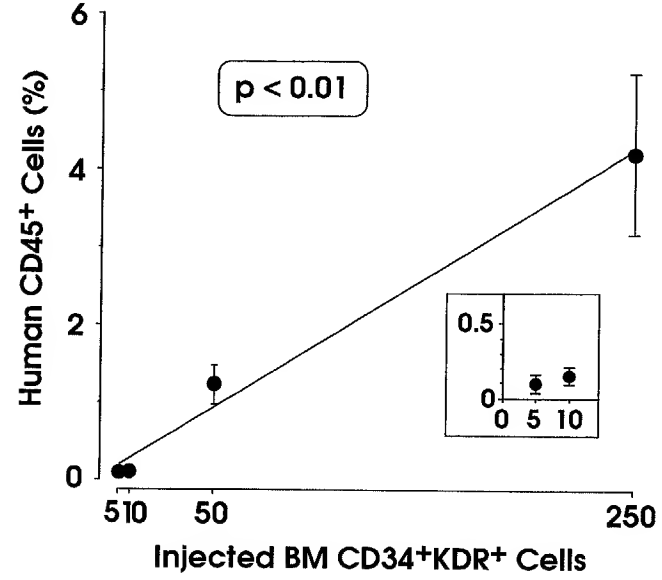
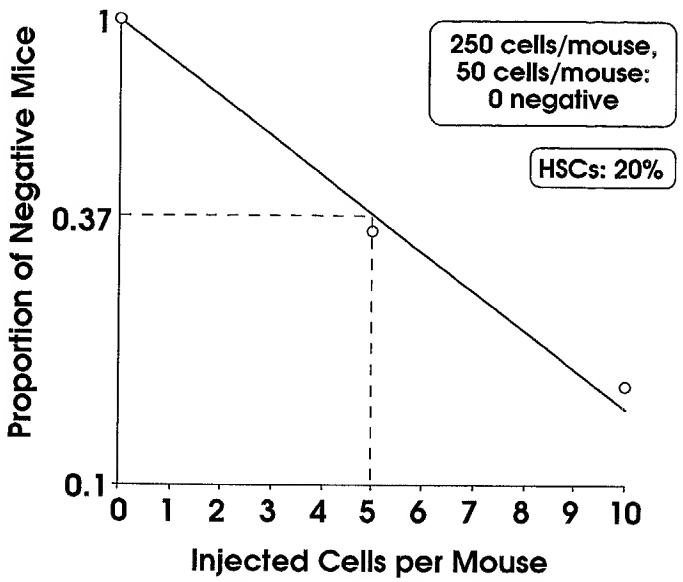
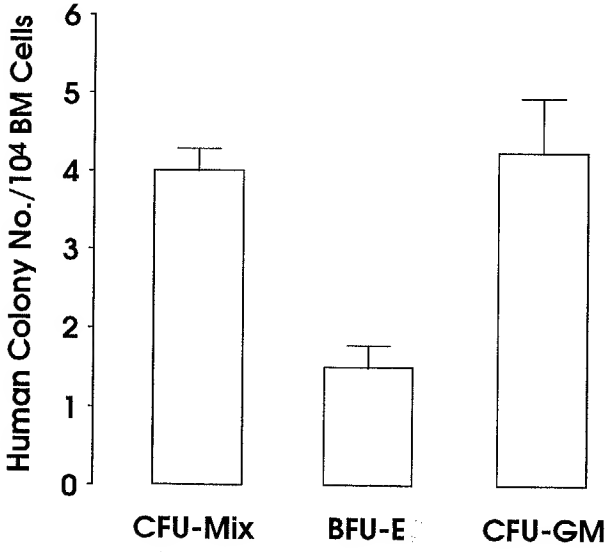

CD34+KDR+
1,600 cells
800
400
100
0.1
11.05
8.62
0.06
7.28
6.14
0.08
4.54
5.31
0.07
1.39
2.77
CD34 PE
CD45 FITC
CD34+
250,000
0.28
3.19
3.51
CD34+KDR-
0.06
0
0.01
p < 0.01
Human CD45+ Cells (%)
Injected BM CD34+KDR+ Cells
CD34+KDR+
1,600 cells
8.93
2.23
0.22
CD33 PE
CD15 FITC
1.90
4.17
23.45
CD14 PE
CD45 FITC
10.54
0.71
1.06
CD71 PE
GPA FITC
2.64
2.14
25.85
CD41 PE
CD45 FITC
3.11
1.09
0.01
CD20 PE
CD19 FITC
4.27
5.93
0.37
CD4/CD8 PE
CD3 FITC
5.07
0.69
0.38
CD56 PE
CD16 FITC

Fig.3A


p < 0.01
Human CD45+ Cells (%)
6
4
2
0
0.5
0
0 5 10
5 10 50 250
Injected BM CD34+KDR+ Cells


250 cells/mouse,
50 cells/mouse:
0 negative
HSCs: 20%
Proportion of Negative Mice
1
0.37
0.1
0 1 2 3 4 5 6 7 8 9 10
Injected Cells per Mouse


Human Colony No./10⁴ BM Cells
6
5
4
3
2
1
0
CFU-Mix
BFU-E
CFU-GM

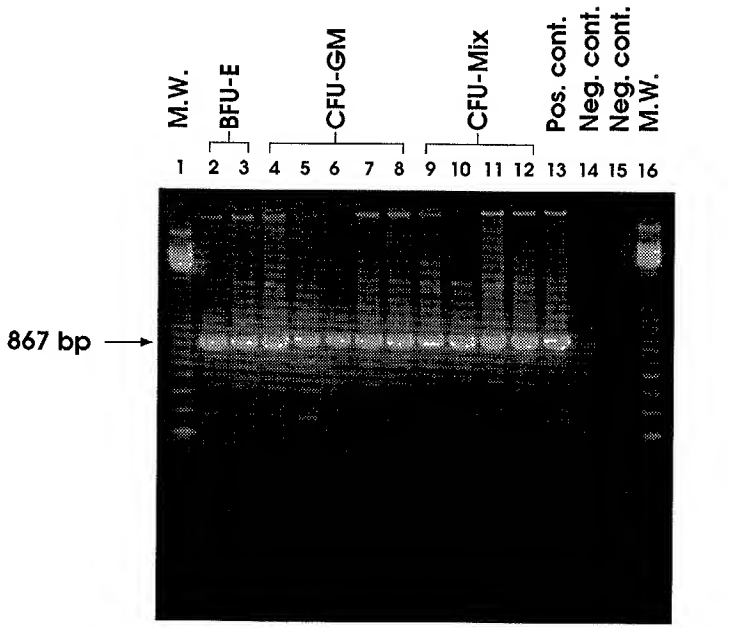

M.W.
BFU-E
CFU-GM
CFU-Mix
Pos. cont.
Neg. cont.
Neg. cont.
M.W.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
867 bp

Fig. 3B

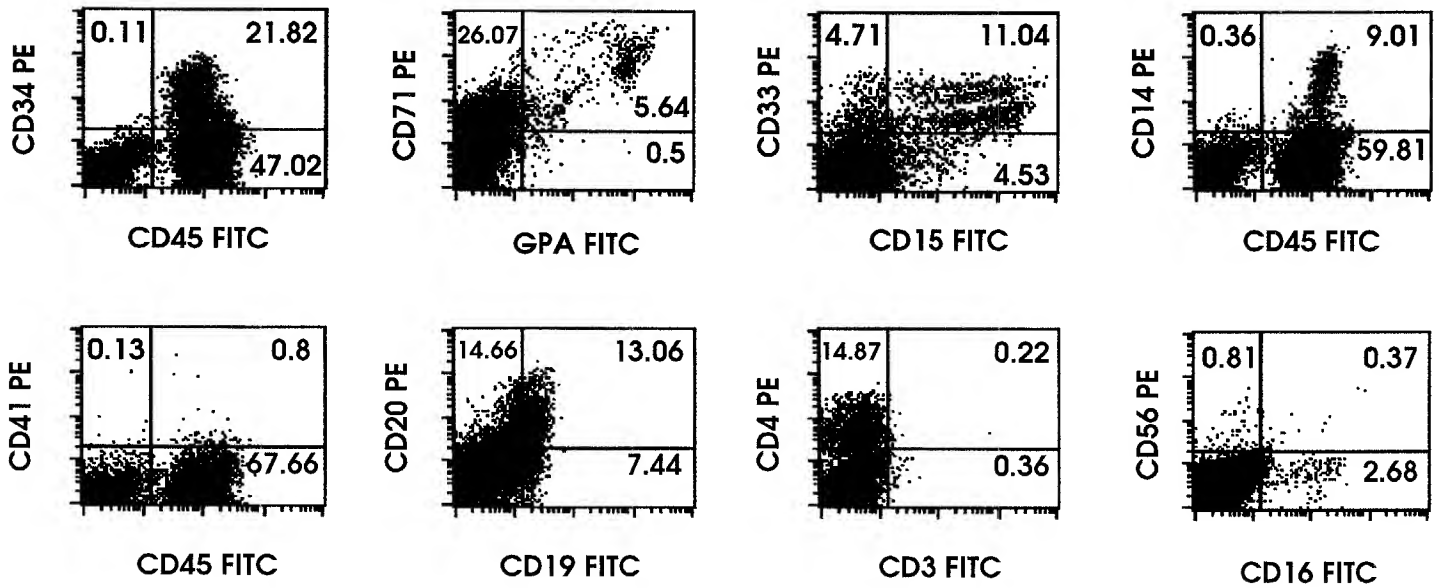

CD34 PE
0.11
21.82
47.02
CD45 FITC
CD71 PE
26.07
5.64
0.5
GPA FITC
CD33 PE
4.71
11.04
4.53
CD15 FITC
CD14 PE
0.36
9.01
59.81
CD45 FITC
CD41 PE
0.13
0.8
67.66
CD45 FITC
CD20 PE
14.66
13.06
7.44
CD19 FITC
CD4 PE
14.87
0.22
0.36
CD3 FITC
CD56 PE
0.81
0.37
2.68
CD16 FITC

Fig.3C

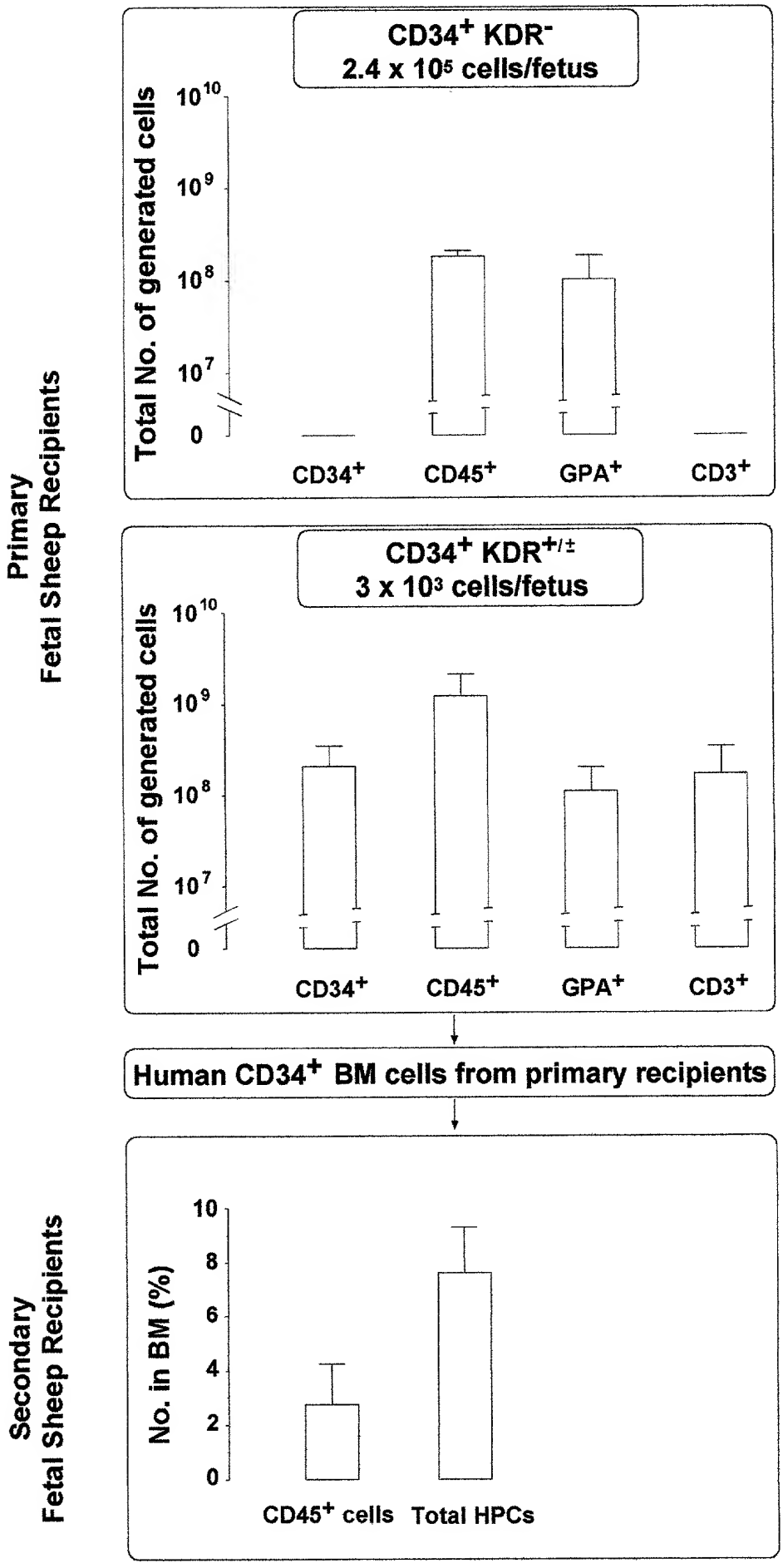

Primary
Fetal Sheep Recipients
CD34+ KDR-
2.4 x 10^5 cells/fetus
Total No. of generated cells
10^10
10^9
10^8
10^7
0
CD34+
CD45+
GPA+
CD3+
CD34+ KDR+/±
3 x 10^3 cells/fetus
Total No. of generated cells
10^10
10^9
10^8
10^7
0
CD34+
CD45+
GPA+
CD3+
Human CD34+ BM cells from primary recipients
Secondary
Fetal Sheep Recipients
No. in BM (%)
10
8
6
4
2
0
CD45+ cells
Total HPCs

Fig. 4

Attorney Docket No. **9855-26U1 (OTT-3038-1)**

DECLARATION AND POWER OF ATTORNEY
(Related Application)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

COMPOSITIONS AND METHODS FOR USE IN AFFECTING HEMATOPOIETIC STEM CELL POPULATIONS IN MAMMALS

the specification of which is attached hereto and/or was filed on May 28, 1999 as Application No. _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d), of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

FOREIGN PRIORITY APPLICATION(S)

			Priority Claimed
			[ ] Yes[ ] No
(Number)	(Country)	(Day/month/year filed)	
			Priority Claimed
			[ ] Yes [ ] No
(Number)	(Country)	(Day/month/year field)	

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional patent application(s) listed below and have also identified below any United States provisional patent application(s) having a filing date before that of the application on which priority is claimed.

PROVISIONAL PRIORITY PATENT APPLICATION

		Priority Claimed
60/087,153	**May 29, 1998**	[X] Yes [ ] No
(Application No.)	(Filing Date)	

I hereby claim the benefit under Title 35, United States Code, Section 120, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or in the prior U.S. provisional application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56, which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status)--(patented, pending, abandoned)

(Application Serial No.)	(Filing Date)	(Status)--(patented, pending, abandoned)

And I hereby appoint the registered attorneys and agents associated with Panitch Schwarze Jacobs & Nadel, P.C., Customer No. 000570, as my attorneys or agents with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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